Identification of signaling pathways important for Borrelia burgdorferi-elicited IL-10 production by macrophages and their effects on suppressing antigen presenting cell immune responses

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Entitled

Identification of Signaling PathwaysImportant for Borrelia burgdorferi-elicited IL-10 Production by Macrophages and Their Effects on Suppressing Antigen Presenting Cell Immune Responses

By

Yutein (Andy) Chung

Submitted to Graduate Faculties as partial fulfillment of the requirements for the Doctor of Philosophy degree in Biomedical Sciences

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August 2011
An abstract

entitled

Identification of signaling pathways important for *Borrelia burgdorferi*-elicited IL-10 production by macrophages and their effects on suppressing antigen presenting cell immune responses

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*Borrelia burgdorferi* (Bb) is a tick-borne bacterium from the family *Spirochaetes* that is the causative agent for Lyme disease. These bacteria are notable for their ability to evade host defenses and persist extra-cellularly, even though infection elicits potent innate and adaptive immune responses. We previously demonstrated that host interleukin 10 (IL-10), an anti-inflammatory cytokine important for controlling excess inflammation, plays an important role in suppressing the immune-clearance of Bb. We hypothesize antigen-presenting cells (APCs) such as bone-marrow macrophages (BMM) and dendritic cells (BMDC) produce high-levels of IL-10 immediately upon recognition of Bb and this dysregulated IL-10 level subsequently suppresses the elicitation of pro-inflammatory mediators by the APCs against Bb. We also hypothesize that the production of IL-10 by APCs such as BMMs utilizes signaling pathways that are distinct from Bb-elicited pro-inflammatory mediators. Our results demonstrated that both cultured BMM and BMDCs rapidly produce IL-10 upon Bb-stimulation and this IL-10 suppressed the production of pro-inflammatory cytokines (e.g. IL-12), chemokines, reactive oxygen species, phagocytosis, and surface marker upregulation. Our data also indicate that IL-10
production by BMMs in response to Bb is dependent on surface Toll-like receptor 2 (TLR2) yet independent of Bb phagocytosis/internalization. While most Bb-elicited pro-inflammatory mediators are also TLR2-dependent, they require that Bb be internalized. Bb-elicited IL-10 production by BMMs is dependent on signaling pathways involving both phosphotidylinositol-3 kinase (PI3-kinase) and mitogen-activating protein kinase (MAP kinase). On the other hand, the elicitation of most pro-inflammatory responses from BMMs by Bb is independent of both PI3-kinase and MAP kinase. Overall, our findings indicate that Bb stimulates APCs to produce dysregulated IL-10 through unique signaling pathways from those that produce inflammatory mediators and that the amount of IL-10 that are produced are sufficient to suppress many APC immune mechanisms that are critical for controlling bacterial infections. The delineation of these IL-10 specific signaling mechanisms should identify pathways that could be targeted to better control the development of Lyme disease.
Dedication

For all the people God has placed in my life, whether past, present, and in the future, that has helped me grow along the way, both in science and in life. My prayers are with you all.
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Chapter 1

Literature Review

1-1: Overview of Lyme disease and its causative agent, *Borrelia burgdorferi*

**Lyme disease:** Lyme disease (LD) was originally documented in Old Lyme, Connecticut in 1976 as a form of juvenile rheumatoid arthritis (Steere, Coburn et al. 2004). It was first characterized as a vector-borne illness in 1977 that is spread by ticks from the genus *Ixodes* (*Ixodes scapularis* in the eastern United States, *Ixodes pacificus* in the western United States, *Ixodes ricinus* in Europe and *Ixodes persulcatus* in Asia) (Steere, Coburn et al. 2004). The true causative agent for LD is the tick-borne spirochetal bacteria *Borrelia burgdorferi* (Bb) (Burgdorfer, Barbour et al. 1982). Today, LD is the leading vector-borne infectious disease in the United States and Europe as well as becoming more prevalent in Asia. In addition, reported cases of LD by the CDC in the United States alone rose from 18,000 to over 23,000 from year 2001 to 2006 (Bacon, Kugeler et al. 2008). Within United States, LD is most prevalent in the northeastern/New England region and Midwest region of the United States with sporadic cases seen near the Pacific Ocean coastline.
The clinical symptoms of LD are usually divided into an early stage and a latent stage. The early stage, which mostly occurs within a few days after a person receives a tick bite, is usually associated with fever, malaise, and cold-like symptoms (Steere 1989; Wilske 2005). The early stage of LD is usually associated with a characteristic erythema migran (EM) (Dandache and Nadelman 2008), or bulls-eye rash near the site of the tick bite. These early symptoms, which often last up to eight days, are typically self-limiting and dissipate. However, if the disease is not diagnosed or treated properly, there is a latent/secondary stage where the LD-causing bacteria Bb re-emerges after months/years of the tick bite. During this latent stage, the patient often experiences severe tissue-specific swelling, pain, and inflammation. Examples of latent stage LD include arthritis (commonly referred to as Lyme arthritis), endocarditis, and neuroborreliosis (e.g. Bell’s palsy, a type of facial paralysis) (Nocton and Steere 1995; Wormser 2006). Although in general LD is not life-threatening, the disabilities resulting from the latent disease, along with the rising number of cases per year is still a major health concern across the nation.

The clinical diagnosis of LD usually begins with determining whether the patient has visited endemic regions, as well as whether they recall receiving a tick-bite (Bratton, Whiteside et al. 2008). EM that is 5 cm or greater near the site of the tick bite indicates Bb infection (Bratton, Whiteside et al. 2008). Otherwise, a CDC recommended two-step serum test to diagnose LD is performed. The first step involves a highly-sensitive enzyme-linked immunosorbent assay (ELISA) that is used to detect Bb-specific antibodies. The ELISA method alone, due to its sensitivity and broad specificity, may often lead to false positives in reporting Bb-infection. Therefore, a more specific detection technique using Western blot to detect Bb-specific antibodies is performed.
following the ELISA (Wilske 2005; Bratton, Whiteside et al. 2008). If LD is diagnosed early through the positive identification of a Bb infection, proper antibiotics such as amoxicillin or doxycycline can be administered orally to clear Bb from the host as well as prevent the development of the later stages of LD (Girschick, Morbach et al. 2009). Even when LD is diagnosed during the later stages, antibiotics such as ceftriaxone administered intravenously can still be used to effectively clear the bacteria (Bratton, Whiteside et al. 2008; Girschick, Morbach et al. 2009). Issues involving the diagnosis of LD include that, with the exception of the characteristic EM, the symptoms of early stages of LD resembles the common cold and usually dissipates within days. This can result in Bb-infected individuals not getting the proper diagnosis/treatment if EM is not present or neglected during the clinical visit. In addition, since the late-stage symptoms often resemble other ailments (e.g. rheumatoid arthritis) and occur months after the tick-bite, it is difficult to connect these symptoms to LD. These issues with LD often lead to clinical misdiagnosis where millions of health care dollars are lost on ineffective therapeutics. Therefore, it is important to understand the causative agents of LD and how they interact with the host organisms in order to optimally design treatments for LD.

**Borrelia burgdorferi, the causative agent of LD:** In 1977, Dr. Allen Steere and colleagues first characterized Lyme arthritis, a common pathology of LD, to be an unusual form of arthritis possibly caused by arthropod vectors (Steere, Malawista et al. 1977). In the early 1980’s, Dr. Willy Burgdorfer and colleagues isolated a tick-borne bacterium from patients of LD (Burgdorfer, Barbour et al. 1982). This bacterium, subsequently named *Borrelia burgdorferi* (Bb) sensu stricto, is the main causative agent
for LD in the United States. In Europe, LD is mainly caused by *Borrelia garinii* and *Borrelia afzelii*; both are also known as Bb sensu lato (Tilly, Rosa et al. 2008). Bb is a Gram-negative spirochetal bacterium of the phylum Spirochaetes, which encompasses the genus *Borrelia* and *Treponema*, as well as the family *Leptospiracaete* that are all known to cause human diseases (Tilly, Rosa et al. 2008). The defining characteristics of the Spirochaetes are that they are spiral-shaped bacteria containing membrane-bound endoflagella (or periplasmic flagella) instead of external flagella, which give these organisms the characteristic wave-like movements. As for the members of the *Borrelia* genus, another unique characteristic is an outer membrane containing mainly tri-acylated lipoproteins instead of the typical lipopolysaccharide or lipo-oligosaccharides found on the surface of Gram-negative bacteria (Takayama, Rothenberg et al. 1987). Bb also is an obligate parasite that is found only within certain host animals. Since Bb is a tick-borne pathogen, its natural entry into host is through tick-bites.

The Bb genome consists of a 0.9 mega-base linear chromosome that comprises 60 percent of its genome, along with multiple copies of both linear and circular extra-chromosomal plasmid DNAs that comprises the other 40 percent (Barbour 1989; Ferdows and Barbour 1989; Casjens, Palmer et al. 2000; Fikrig and Narasimhan 2006; Tilly, Rosa et al. 2008). The chromosomal DNA of Bb contains prototypical bacterial genes important for metabolism, replication, and motility (Casjens, Palmer et al. 2000). However, Bb lacks many genes important for biosynthesis of essential nutrients, which explains the requirement for Bb to acquire essential nutrients from living animal hosts (Casjens, Palmer et al. 2000). There are at least 21 linear and circular plasmids found in Bb that is almost exclusively involved in expression of their surface lipoproteins.
(Casjens, Palmer et al. 2000). Genes present on Bb plasmids display evidence that they are in a state of rapid evolution such that many genes were either duplicated and/or inactivated through mutations (Tilly, Rosa et al. 2008). The general composition of the Bb surface lipoproteins has been well-characterized. The lipid portions of Bb lipoproteins are usually conserved structurally. It contains an N-terminal tri-acylated tripalmitoy-S-glyceryl cysteine (PAM₃Cys) motif with host-derived fatty acid chains buried within the bacterial membrane (Brandt, Riley et al. 1990). On the other hand, the protein portion, which is exposed to the external environment, is highly variable. There are over 130 different genes that appear to encode lipoproteins, with only a few having been described to have some virulence capacity, such as attachment to tick-midgut or evasion of host immunity. Since Bb lipoproteins are predominantly encoded by their plasmids, they can be rapidly altered in response to environmental changes (Fikrig and Narasimhan 2006). For example, the outer surface protein A (OspA), one of the first Bb lipoproteins identified, is expressed at high levels on Bb while they dwell in the midgut of the tick (Barbour 1989; Yang, Pal et al. 2004). As Bb moves from the tick midgut to the salivary gland during the process of transfer from tick to mammal, the level of OspA is down-regulated while another lipoprotein, OspC, is upregulated (Pal, Yang et al. 2004; Fikrig and Narasimhan 2006). Immediately following the entry into mammalian hosts, Bb lipoproteins that function to evade host immune responses are up-regulated (Fikrig and Narasimhan 2006). The proposed functions of some of these Bb lipoproteins will be described in later sections.
**Host range and transmission cycle of Bb:** As an obligate host parasite, Bb must be able to adapt to living in various host animals. Bb is known to actively infect ticks and vertebrate hosts such as humans, deer, mice, and birds (Levine, Wilson et al. 1985; Brown and Lane 1992; Tilly, Rosa et al. 2008). Among these hosts, small rodents such as the white-footed mouse serve as the natural or reservoir hosts for Bb due to an optimal symbiotic relationship between host and Bb; these hosts allow Bb to survive long-term and be propagated to subsequent hosts (Brown and Lane 1992). On the other hand, humans and deer serve as terminal hosts for Bb, thus resulting in a low possibility that Bb can be transmitted again. Due to a poor symbiotic relationship between Bb and host, terminal hosts are far more susceptible to develop disease (such as LD) when infected with Bb as compared to reservoir hosts. Although pathologies resulting from Bb infection rarely leads to death of the infected host, the symptoms, such as arthritis, that develop can quickly hinder the movements of the host, reducing their likelihood to survive in nature (i.e. less likely to escape from predators).

Understanding the tick life cycle and its relationship to the infection cycle for Bb is important in understanding the etiology of LD. During the summer, larval ticks hatch from eggs that are usually sterile of Bb. As the summer season ends, larval ticks usually take a blood meal from vertebrate hosts and subsequently molt to nymph. During this process, the tick may acquire Bb if the blood meal is taken from an infected animal. The infected nymph tick must take another blood meal before they molt to adulthood the following spring, thus allowing Bb to be transferred again to a different host (Steere, Coburn et al. 2004). Finally, adult female ticks take a blood meal during autumn before laying eggs, which will hatch the summer of the following year (Steere, Coburn et al.)
Due to this seasonal feeding of the ticks, LD prevalence is usually the highest during times when ticks take blood meal (e.g. late spring/summer) (Steere, Coburn et al. 2004; Wormser 2006). Since ticks take blood meal only one to two times a year, Bb is required to persist within the same host for up to a year before the next tick feeding cycle takes place.

The ability of Bb to persist long-term in the infected animal host plays a major role in the pathophysiology of LD. For mammals such as humans, Bb tends to persist in tissues such as joint, heart, and brain; this is consistent with the observed pathology of LD (Yoshinari, Steere et al. 1989; Nocton and Steere 1995; Steere, Coburn et al. 2004). Mammals also possess a multi-tier immune system that the bacteria must overcome in order to persist within that host. Therefore, in order to better understand mechanisms of Bb persistence in host organisms, it is important to first study how the host immune system interacts with Bb, both in animal models and at the cellular level. Ultimately, the translation of this knowledge into the clinical setting will provide better therapeutic options for LD.

**Culture media and Bb in the laboratory:** Studies indicate that mice, hamster, and rhesus monkeys can all serve as animal models for both the study of the pathogenesis of LD and host-Bb interactions *in vivo* (Philipp and Johnson 1994; Wooten and Weis 2001). On the other hand, studies performed *in vitro* are challenging since Bb is an obligate parasite and requires nutrients not found in conventional culture media for bacterial growth. The only known culture media that supports the growth of Bb outside of a living host was developed by Barbour et al. in 1984, and is named Barbour, Stoenner, Kelly or
BSK media (Barbour 1984). Since then, this nutrient-rich media has been modified and tested for the optimal growth of Bb (Pollack, Telford et al. 1993). The advent of a Bb growth media allows the propagation of Bb and the ability to perform animal infection experiments without relying on tick vectors to transmit the bacteria from host to host; this allows better experimental design for the study of Bb and its interactions with host animals. Therefore, the development of the BSK media has been important for advancements of knowledge on how the host immune system interacts with Bb.
1-2: Immune system and Bb

**Innate immunity I, TLRs and inflammation:** In order for pathogens such as Bb to establish an infection, it first needs to overcome the immediate barriers of the host. These barriers, known as innate immunity, usually start at the skin, where the thick epidermal layer fortifies the host from pathogen entry. Mammalian cells also express an abundant amount of pattern-recognizing receptors (PRRs) on their surface capable of recognizing pathogen-associated membrane protein (PAMPs), resulting in the propagation of intracellular responses toward the stimuli (Janeway and Medzhitov 2002; Taylor, Martinez-Pomares et al. 2005). One example of PRR is the toll-like receptor (TLR) family, the mammalian homologues to the toll receptors in *Drosophila* (Medzhitov 2001). These receptors are characterized by the leucine-rich repeat domains present on the extracellular region and the toll-interleukin 1 receptor (TIR) domain on the intracellular region (Kumar, Kawai et al. 2009). There are at least 9 known members of TLR family in human and 11 in mice, and each member recognizes various microbial ligands (Pasare and Medzhitov 2005; Beutler 2009; Kumar, Kawai et al. 2009). Examples of surface TLRs and their ligands include TLR2, TLR4, and TLR5 for the recognition of the peptidoglycan cell wall, lipo-poly or lipo-oligo saccharide cell wall, and bacterial flagella, respectively. TLRs are also known to be expressed intracellularly within the membrane-bound endosomes. These intracellular TLRs include TLR3/7 and TLR8 for recognition of double-strand RNA, and TLR9 for recognition of unmethylated CpG DNA motifs (Kumar, Kawai et al. 2009).
Upon recognition of their respective ligands, cell surface TLRs such as TLR 2 and 4 will recruit the adaptor protein myeloid differentiation factor 88 (MyD88) to the TIR domain of the TLRs through the bridging activity of TIR activation protein (TIRAP) (Uematsu and Akira 2006; Kumar, Kawai et al. 2009). The recruitment of MyD88 to the TIR domain further recruits other signaling molecules, such as interleukin-1 receptor associated kinases (IRAKs) and TNF receptor-activation factor (TRAF) members. The ultimate downstream effector of TLR signaling is the activation of nuclear factor kappa B (NF-κB), a transcription factor important for turning on various mediators of host defense and inflammatory responses (Lenardo and Baltimore 1989; Hayden, West et al. 2006; Ghosh and Hayden 2008). The NF-κB complex is most commonly composed of a p50 and a p65 (or RelA) subunit that is known to bind DNA. When the cells are quiescent, the p50/p65 subunits of NF-κB are sequestered from the nucleus and remain in the cytosol through the binding of an inhibitor of kappaB (IκB) protein. Downstream TLR signaling cascades result in the phosphorylation of Iκ kinase (IKK) which in turn phosphorylates the IκB protein. Phosphorylated IκB is ubiquitinated and targeted for proteasome degradation; resulting in the release of p50, p52, p65 (RelA), RelB, c-Rel subunits of NF-κB that can now enter the nucleus to begin transcription of various immune-effector genes (Hayden, West et al. 2006; Ghosh and Hayden 2008).

NF-κB activation results in the elicitation of an inflammatory response (or inflammation), which is an important component of innate immunity and host defense against pathogens. During an infection with a bacterial pathogen (e.g. Bb), the main functions of inflammation are to eliminate or clear the pathogen from the host through the recruitment of circulating leukocytes to the site of infection, prevent the spread of
infection throughout the body by inducing blood clots, and to repair damaged tissues that occurred during the process of host defense (Janeway 2009). The characteristics of inflammation are heat (calor), pain (dolor), swelling (tumor), and redness (rubor) at the local site of infection, along with fever and enlarged lymph nodes (Janeway 2009).

Among the genes activated by NF-κB that are important for initiation of inflammation are chemical hormones of the immune system known as cytokines. Key cytokines produced during the early stage of inflammation are tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1), both of which are known for vasculature dilation, fever induction, activation of leukocytes, and the enhancement of acute-phase protein responses (Pfeffer 2003; Dinarello 2010). Cytokines produced during later stages of inflammation include IL-6, which is known for its ability to induce acute-phase responses as well as activation of certain T lymphocytes (Scheller and Rose-John 2006). IL-12, originally identified as natural killer stimulating factor (NKSF), is another late pro-inflammatory cytokine known for the activation of T lymphocytes, natural killer (NK) cells, and neutrophils (Kobayashi, Fitz et al. 1989; Trinchieri 2003). Besides cytokines, chemokines (or chemo-attractive cytokines) are also key mediators of inflammatory responses that function primarily by recruiting other immune cells to the site of infection through the activation of G-protein coupled receptors. Chemokines can be classified based on the amino acid motif on the N-terminus. The two main classes are the CXC and the CC class where the C stands for cysteine residue and the X is any other amino acids (Matsukawa, Hogaboam et al. 2000; Fernandez and Lolis 2002). Examples of chemokines include CXCL1 (KC in mice), CXCL8 (IL-8 in humans) and CXCL2 (MIP-2) that functions to recruit polymorphonuclear cells, such as neutrophils (DeFilippo,
Other chemokines include CCL3 (MIP1α) and CCL5 (RANTES) which are important for the recruitment of mononuclear cells such as monocytes and T and B lymphocytes (Fernandez and Lolis 2002).

**Innate immunity II, Myeloid leukocytes:** Myeloid leukocytes function as sentinels of innate immunity since they are usually the first set of immune cells in the body to encounter pathogens (Gordon 2004). One of these cell types are macrophages (MØs)(Gordon 2007). Derived from the circulating monocytes within the bloodstream, MØs usually reside within host tissues such as skin, liver, and lung to fend off invading pathogens (Gordon 2004). MØs expresses a high level of PRRs, such as the TLRs, both on the cell surface and within endosomes, where they are capable of recognizing a plethora of foreign pathogens (Underhill and Ozinsky 2002). MØs have the ability to engulf microbial pathogens through the process of phagocytosis; thus, they are also classified as phagocytes. Phagocytosis is initiated when MØ surface receptors (e.g. scavenger receptors or Fc receptors) recognize the pathogen and induce Rho G-protein and actin-dependent invagination of the plasma membrane around the pathogen, allowing for its internalization (Underhill and Ozinsky 2002). Upon internalization of the pathogen, the surrounding plasma membrane evolves into an endosome-like vesicle known as the phagosome. As the phagosome matures, its lumen becomes acidified as it fuses with lysosomes to form a phagolysosome, where the internal pH can be around 4-4.5. (Underhill and Ozinsky 2002; Stuart and Ezekowitz 2005; Luzio, Pryor et al. 2007; Kinchen and Ravichandran 2008). The low pH activates lysozymes within the phagolysosome, which are important for the direct killing and the breakdown of the
This enables the processing of the pathogen into antigens presentable on the cell surface of MØs for the activation of T cells (to be mentioned later) (Saftig and Klumperman 2009). The phagolysosome can also fuse with endosomes containing intracellular TLRs (e.g. TLR9) where further TLR signaling can take place. During phagocytosis, MØs can also produce a respiratory burst; a process where they generate reactive oxygen species (ROS) in high amounts within the phagosomes to augment the killing and breakdown of pathogens (Gordon 2007). One enzyme responsible for ROS generation within MØs is NADPH oxidase, which is composed of membrane-bound proteins gp91phox and p22phox, along with the cytosolic components p47phox, p40phox, p67phox, and Rac-GTPase (Babior 2004). NADPH oxidase catalyzes the formation of superoxide radicals from molecular oxygen (Babior 2004). Besides ROS, MØs can also generate reactive nitrogen species for the killing of intracellular pathogens. The enzyme inducible nitric oxide synthase (iNOS) is upregulated in MØs through the activation of NF-κB. iNOS catalyzes the formation of nitric oxide (NO) from molecular oxygen and guanidino nitrogen of L-arginine (Nathan and Hibbs 1991).

Similar to MØs, dendritic cells (DCs) are also myeloid cells that can take residence in various tissues. Unlike MØs, DCs are less known for direct killing of pathogens and more known for their ability to activate adaptive immunity, usually by internalizing and processing of pathogens, presenting antigens on their surface, upregulating surface co-stimulatory molecules such as CD80 and CD86 (also known as B7-1 and B7-2, respectively), and entering lymph nodes to activate (or prime) naïve T cells (Steinman 2007). Like MØs, DCs can also dwell in different tissues. One example of a tissue-specific DC are the Langerhan cells which dwell in the epidermal layer of the
skin (Merad, Ginhoux et al. 2008). DCs also express PRRs such as TLRs at high levels for the sensing of various PAMPs, and when stimulated will produce a plethora of pro-inflammatory cytokines and chemokines important for the activation and recruitment of other immune cells. Due to their ability to present foreign microbial antigens to the adaptive immune system through major histocompatibility complexes (MHCs), MØs and DCs are also known as resident antigens presenting cells (APCs).

Besides APCs, neutrophils are another class of innate immune leukocyte that are important for pathogen killing. Unlike APCs, resting neutrophils circulate within the bloodstream most of their lifetime. Upon the recognition of microbial proteins or chemokines, neutrophils become activated and migrate toward the source of these activating molecules. Activated neutrophils undergo phagocytosis, respiratory burst, secrete pro-inflammatory cytokines, and release granules containing anti-microbial agents at a rapid rate (Kumar and Sharma 2010). However, neutrophils do not live long within tissues and undergo apoptosis soon after interacting with pathogens (Kennedy and DeLeo 2009). Overall, innate immune leukocytes, including the ones mentioned above, as well as mast cells, eosinophils, and NK cells, work in concert with each other to provide rapid protection against pathogens and to initiate the adaptive immune responses.

**Adaptive immunity:** The adaptive immune system, which is composed of T and B lymphocytes, cooperates with innate immunity in defenses against pathogens. Unlike the innate immune response, the activation of adaptive response is usually slower and can take several days following the onset of an infection. However, adaptive immune responses are much more specific against a certain pathogen and possess immunological
memory. Most resting lymphocytes circulate through bloodstream but can recirculate to secondary lymph organs such as draining lymph nodes and the spleen. Upon sensing and processing of pathogens, APCs will present peptide fragments or microbial proteins (i.e. antigens) bound to MHC class I (MHC I) or MHC class II (MHC II) on the cell surface and migrate toward the lymph nodes. Once in the lymph nodes, APCs will subsequently interact with CD4+ T cells through the binding of T cell receptor (TCR) with MHC II-peptide complex or activate CD8+ T cells through the binding of TCR with MHC I-peptide complex. In addition to TCR binding to MHC II complex, activation of naïve CD4+T cells requires co-stimulatory signals provided by the binding of APCs surface ligands CD80 and CD86 with either T cell surface receptors CD28, which results in T cell activation or Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), which results in T cell suppression. Other molecular interactions at the immunological synapse that are important for T cell activation include the binding of integrin class molecules such as CD54 on APCs with lymphocyte functioning antigen-1 (LFA-1) on T cells (Dustin 2008; Janeway 2009). Activated CD4+T cells can further differentiate into T-helper 1 (Th1) which is known to aid in cell-mediated immunity. Some CD4+ T cells can differentiate into T-helper 2 cells (Th2) that are important for mediating humoral responses or T-regulatory cells (Tregs) that negatively regulates immune responses. Like innate immune cells, activated T lymphocytes are also capable of secreting cytokines. For example, Th1 lymphocytes secrete interferon gamma (IFN-\(\gamma\)) which functions to enhance cell-mediated killing of pathogens by phagocytes through activities such as enhancement of respiratory burst and antigen presentation (Schroder, Hertzog et al. 2004; Teixeira, Fonseca et al.)
on the other hand, Th2 lymphocytes secrete IL-4, which is important in mediation of humoral responses.

B lymphocytes are most important for the secretion of immunoglobulins (Ig) molecules, also known as antibodies. The antibodies produced have multiple functions in immune responses against foreign pathogens. The variable, or Fab region of antibodies can recognize and bind a variety of antigenic molecules (i.e. epitopes) on the surface of foreign pathogens. Upon antigen binding, the Fc region can bind to receptors expressed on APCs, neutrophils, and other phagocytes of the innate immune system. This process, known as opsonization, greatly enhances the phagocytic intake and killing of foreign pathogens. Antibodies also can initiate complement activation from the classical pathway (to be mentioned later) and can also function to directly neutralize certain bacterial toxins (Janeway 2009).

Other important immune effectors: Acute-phase proteins that are mainly produced and secreted by the liver can also play a key role in immunity. The best known acute-phase proteins are members of the complement system, a series of proteins/enzymes that can recognize foreign pathogens and mediate their killing both directly and indirectly. There are three major complement pathways: classical, lectin, and alternative, that all ultimately lead to the deposition of complement component C3b on the surface of pathogens (Tomlinson 1993; Janeway 2009). The classical and the lectin pathways require either antibodies or mannose lectin proteins to first bind to the pathogen’s surface before C3b can be deposited. The alternative pathway, on the other hand, can spontaneously fix C3b from the pathogen surface (Tomlinson 1993). Accumulation of
C3b will recruit binding of C5b on the pathogen surface and began a cascade of complement protein binding (i.e. C6, C7, C8, and C9) which ultimately lead to the formation of the membrane attack complex that directly lyses the membrane and kills the pathogen. In addition, C3b can act as an opsonin and bind membrane receptors that enhance the intake and killing of the pathogen by phagocytes. The complement pathways can also generate chemotactic peptides, such as C5a, that function as a pro-inflammatory mediator to recruitment and activate leukocytes (Guo and Ward 2005). To protect the host from complement-mediated damage, host membranes contain negative regulators of complement activation, such as CD59 and factor H, that inhibit the formation of the membrane attack complex. Overall, the complement system enhances the cellular immune responses by killing pathogens either directly or by cooperating with innate and adaptive immune mediators.

**Host immune responses against Bb:** Identifying the critical interactions between Bb and the host immune response is pivotal for understanding how this bacteria persists and causes disease within an immunocompetent, but susceptible host (Seiler and Weis 1996). The many lipoproteins expressed on the surface of Bb are known to be highly immunogenic (Brandt, Riley et al. 1990). Upon infection, Bb activates resident APCs such as MØs within the skin through the recognition of Bb lipoproteins (Salazar, Pope et al. 2005). Bb lipoprotein is also known to activate the NF-κB pathway, which subsequently leads to the production of various pro-inflammatory cytokines and chemokines (Wooten, Modur et al. 1996). Bb lipoproteins also can prime neutrophils, resulting in upregulated surface molecules (e.g. CD11b) and enhanced responses against
subsequent stimulation by N-formyl peptides (Morrison, Weis et al. 1997). In addition, *in vitro* studies demonstrate that MØs and neutrophils readily take up and kill Bb through phagocytosis (Montgomery and Malawista 1996; Montgomery, Lusitani et al. 2002). The elicitation of innate immune responses by Bb through its lipoproteins is responsible for the fever and inflammation during the early stages of LD (Seiler and Weis 1996).

Currently, the best known host cell receptor that recognizes Bb is TLR2. TLR2 is known to form a signaling complex with either TLR1 or TLR6 on the cell surface and associates with adapter protein MyD88 intracellularly (Takeda, Kaisho et al. 2003). TLR2 complexes can recognize a variety of microbial surface molecules, including peptidoglycan, lipoteichoic acid, yeast zymosan, and a variety of lipoproteins (Aliprantis, Yang et al. 1999; Lien, Sellati et al. 1999; Schwandner, Dziarski et al. 1999). Although Bb possesses a minimal peptidoglycan cell wall and lacks lipoteichoic acid, the PAM₃Cys motif of Bb lipoproteins is potent agonists for TLR2 (Aliprantis, Yang et al. 1999). *In vivo* studies indicate that TLR2 is required for the Bb-elicited pro-inflammatory cytokine production by the immune cells (Hirschfeld, Kirschning et al. 1999). Recombinant Bb-lipoproteins have also been shown to elicit cytokine production from macrophages *in vitro* through TLR2 signaling (Wooten, Ma et al. 2002). Studies also suggest that the cooperation between TLR2 and TLR1 is required for the optimal recognition of Bb lipoproteins on cell surfaces (Alexopoulou, Thomas et al. 2002). Among all innate immune receptors, TLR2 seems to be the most important in recognizing Bb.

Besides innate immunity, Bb can also elicit a potent adaptive immune response. Animals studies indicate that Bb-specific antibodies can be detected within infected hosts
a few days post-Bb infection (Barthold and Bockenstedt 1993). Clinical studies also revealed the presence of both Bb-specific antibodies and Bb-specific T cells against OspA in human LD patients (Shanafelt, Anzola et al. 1992). Cytokines such as IFN-γ and IL-4 which are characteristics of Th1 and Th2 responses, respectively, were up-regulated in Bb-infected mice (Keane-Myers and Nickell 1995; Anguita, Thomas et al. 2001) and in human Lyme arthritis patients (Widhe, Jarefors et al. 2004). Studies also indicated that mice infected with Bb demonstrated an upregulation of T cell-recruiting chemokines (e.g. CXCL-10) and a corresponding increase in T cell numbers in Bb-containing tissues (i.e. ankles) after two weeks of infection. (Wang, Ma et al. 2008).

**Evasion of host immunity by Bb:** Despite eliciting a very potent immune response, the clearance of Bb from susceptible hosts is usually incomplete, leading to long-term Bb persistence. Even though differences between Bb numbers and pathologies exist between Bb-infected wild-type (WT) mice and severe combined immune-deficient (SCID) mice, a mouse that is deficient in functioning T and B lymphocytes (Barthold, Sidman et al. 1992; Schaible, Vestweber et al. 1994; Wang, Ma et al. 2005), adoptive transfer of T cells from pre-infected mice to naïve mice provide very little protection against Bb infection (de Souza, Smith et al. 1993). Specific antibodies are able to prevent Bb infection (Barthold and Bockenstedt 1993; Telford, Kantor et al. 1995), however, antibody-mediated clearance is only effective during the very early stages of infection (Barthold, Sidman et al. 1992; Barthold and Bockenstedt 1993). Adaptive immune cytokines such as gamma interferon and IL-4 showed very little effect in promoting Bb clearance
Overall, adaptive immunity has only a limited effect on Bb clearance. Current literature indicates that innate immunity tends to play a bigger role in Bb clearance compared to adaptive immunity. Bb-infected mice that lacks a functional TLR2 (TLR2<sup>−/−</sup>) demonstrated significantly greater Bb loads in tissues, such as heart and ankle, than TLR2-competent WT Mice (Wooten, Ma et al. 2002; Wooten, Ma et al. 2002) and SCID mice (Wang, Ma et al. 2005). In fact, lack of TLR2 affected only the innate immune response and not the antibody response against Bb (Wooten, Ma et al. 2002; Wooten, Ma et al. 2002). On the other hand, generation of both ROS and RNS appear generally ineffective in Bb clearance (Seiler, Vavrin et al. 1995; Brown and Reiner 1999; Crandall, Ma et al. 2005). Mice with the beige phenotype, which are deficient in functioning NK cell and certain granulocyte populations, did not show better clearance of Bb over wt mice (Barthold and de Souza 1995). Therefore, despite evidence supporting innate immunity may be more important in Bb clearance than the adaptive response; Bb apparently can still find ways to evade these immune effectors.

Being an obligate host parasite, Bb must possess strategies to resist clearance by the infected host. Most of these strategies rely on the ability of Bb to rapidly shift the expression of its surface lipoproteins once inside the host to evade the immune system (Singh and Girschick 2004; Fikrig and Narasimhan 2006). The aforementioned example of how Bb shifts from having OspA to OspC as its dominant surface lipoprotein as they transition from ticks to mammal, illustrates how rapidly Bb shift their lipoproteins in response to changes in environment (Fikrig and Narasimhan 2006). Other studies indicate that immediately after the transition from tick vector to mammalian host, Bb
rapidly expresses lipoproteins that are distinctive from those when it was in the tick (Suk, Das et al. 1995). While in the mammalian host, Bb continues to shift the expression profile of its lipoproteins. As a result, the specific antibodies produced against Bb surface lipoproteins can quickly be rendered ineffective. This rapid shifting of surface antigens by invading pathogens is known as antigen variation, a common immune-evasive strategy employed by many vector-borne pathogens including Bb (Barbour and Restrepo 2000; Singh and Girschick 2004). Certain Bb lipoproteins, such as OspC and VlsE, are variably expressed to provide extensive antigen variation (Zhang, Hardham et al. 1997; Singh and Girschick 2004). Other Bb lipoproteins can directly interfere with host immune mechanisms. These include members of the Erp and CRASP family, which can directly bind factor H from the host and thus prevent activation of the alternative complement pathway and the deposition of C3b on the bacterial surface (Kraiczy, Hartmann et al. 2004; Kraiczy, Hellwage et al. 2004). Bb lipoprotein OspB has also demonstrated the ability to suppress neutrophil activation (Hartiala, Hytonen et al. 2008). Bb in general is believed to be less susceptible to ROS damage than other bacteria since it does not require iron for growth (Posey and Gherardini 2000). However, Bb still expresses superoxide dismutase, which quickly converts the highly reactive superoxide anion to less reactive hydrogen peroxide (Esteve-Gassent, Elliott et al. 2009). During the transmission of Bb from tick to mammal, Bb lipoprotein OspC can sequester Salp15, a protein component from tick saliva, immediately prior to entry into mammalian hosts (Singh and Girschick 2004; Fikrig and Narasimhan 2006). Salp15 has been demonstrated to down-regulate T cell activation in the mammalian host and may aide Bb in the evasion of host immunity (Anguita, Ramamoorthi et al. 2002).
The host immune system contains many mediators to defend and clear a variety of invading pathogens from our body. However, Bb has also adapted many strategies to counteract against these immune mediators within the susceptible host. This partially explains why many key immune mediators that are important for clearance of many other bacteria are ineffective against Bb. However, recent advancements in the field suggested that the host immune-regulatory cytokine interleukin-10 (IL-10) plays a significant role in Bb clearance. Therefore, understanding the effect of IL-10 on the immune mediators against Bb and how IL-10 is produced by the host in response to Bb infection will be beneficial for the development of effective immune-based therapeutics toward better clearance of Bb and prevent the development of latent LD pathologies in an infected host.
1-3: IL-10 and its role in Bb clearance

Overview of IL-10: IL-10 is a cytokine that functions to negatively control immune responses and excess inflammation (Moore, de Waal Malefyt et al. 2001; Couper, Blount et al. 2008). IL-10 is one of the best-described among many other regulatory/suppressive cytokines, including IL-22 and transforming growth factor beta (TGF-B) (Mosser and Zhang 2008; Sanjabi, Zenewicz et al. 2009). The function of IL-10 as an anti-inflammatory cytokine was first discovered when mice that are genetically deficient of IL-10 (IL-10−/−) were observed to develop inflammatory bowel disease (IBD) more readily than wild-type mice (MacDonald 1994). IL-10 has been demonstrated to down-regulate a variety of immune responses, including Th1 T cell differentiation and activities, production of pro-inflammatory cytokines (e.g. TNF and IL-12), and various neutrophil activities (Bazzoni, Tamassia et al. 2010). For myeloid cells such as APCs, IL-10 is known to suppress co-stimulatory molecules such as CD80/CD86 (Mitra, Judge et al. 1995). IL-10 is produced primarily by immune/hematopoietic cells, such as MØs, dendritic cells, neutrophils, B cells, Th2, and Tregs. IL-10 can also be produced by non-hematopoietic, cells such as keratinocytes and epithelial cells (Moore, de Waal Malefyt et al. 2001; Mosser and Zhang 2008; Saraiva and O'Garra 2010).

IL-10 has been associated with a variety of inflammatory diseases clinically. As aforementioned, it was originally discovered for its role in IBD. Deficiency in IL-10 was characteristic for patients that chronically develop IBDs, such as ulcerative colitis and Crohn’s disease (Schreiber, Heinig et al. 1995; Duchmann, Schmitt et al. 1996). The administration of IL-10 via probiotics is currently available and beneficial in the
treatment of IBD (Peluso, Fina et al. 2007). In addition to IBD, IL-10 has also been demonstrated to protect the host from sepsis as well as autoimmune diseases, such as rheumatoid arthritis (Katsikis, Chu et al. 1994; Moore, de Waal Malefyt et al. 2001).

**IL-10 receptors and the effect of IL-10 signaling:** Both immune and non-immune cells have been shown to respond to IL-10. IL-10 is a member of the type II cytokine family, which includes IL-20, IL-22, and IL-28. The IL-10 receptor primarily consists of heterodimers of IL-10R1 and IL-10R2, where the receptor tyrosine kinase JAK1 and Tyk2 were associated with IL-10R1 and IL-10R2, respectively (Yoon, Logsdon et al. 2006; Mosser and Zhang 2008). When the IL-10 ligand binds to the IL-10R1/R2 heterodimers, the receptors dimerize with another IL-10R1/R2 dimer set, therefore creating a tetramer of IL-10 receptors on the cell surface (two sets of each IL-10R1 and IL-10R2) (Mosser and Zhang 2008).

IL-10 binding to its receptor is known to activate the JAK receptor tyrosine kinase pathway, leading to the subsequent phosphorylation of STAT3. Phosphorylation of STAT3 causes it to form homodimers and translocate to the nucleus to turn on various genes. Genes known to be upregulated through the IL-10 receptors include the IL-10 gene itself (positive feedback), as well as several genes known to suppress immune mediators (Mosser and Zhang 2008). One of such example is the suppressor of cytokine signaling (SOCS-3) molecule; a src-homology 2 (SH2) domain-containing protein that can readily bind to other proteins containing phosphorylated tyrosine residues and acts as an E3 ubiquitin ligase in order to target them for degradation by the proteasomes (Murray 2007). As a result, IL-10-mediated SOCS-3 activation down-regulates production of
many pro-inflammatory cytokines or activities, such as IL-6 signaling (Murray 2007). Genes that are turned on by IL-10-induced STAT3 activation can directly suppress the transcription of pro-inflammatory genes via NF-κB induction (Murray 2006). IL-10 signaling can also suppress NF-κB activation by preventing IKK from phosphorylating IKβ; thereby preventing the release of p50/p65 subunits and its entry into the nuclei (Williams, Ricchetti et al. 2004). IL-10 can also suppress immune activities by the down-regulation of MyD88 expression in myeloid cells. (Dagvadorj, Naiki et al. 2008). Studies also indicated that IL-10 blocks APC superoxide production by directly interfering with NADPH oxidase assembly (Dang, Elbim et al. 2006; Qian, Hong et al. 2006). IL-10 has even been reported to induce apoptosis in mast cells through a STAT3 and p53-dependent manner (Bailey, Kashyap et al. 2006). Therefore, IL-10 can attenuate many immune mediators through a plethora of methods.

**Role of IL-10 in infectious diseases:** Artificial elicitation of host IL-10 to suppress immune killing is a known strategy employed by various pathogenic microorganisms to achieve persistence within host. Dysregulated IL-10 production has been associated with infection by multiple species of bacteria, virus, protozoa, and parasitic worms (Moore, de Waal Malefyt et al. 2001; Couper, Blount et al. 2008). Bacteria known to take advantage of host IL-10 include *Yersinia pestis*, a vector-borne (flea) bacterium that is the causative agent in bubonic plague. The V-antigen LcrV of *Yersinia* can induce host IL-10 in the host and induction is associated with increased bacterial loads (Nedialkov, Motin et al. 1997; Brubaker 2003). Non-vector borne bacteria, such as *Bordetella pertussis*, utilizes their toxin CyaA to induce host IL-10 while suppressing IL-12 (Ross, Lavelle et al.
Besides bacteria, certain viruses can also utilize host IL-10 for their persistence. These includes Hepatitis C virus, which utilizes non-structural protein 4 (NSV4) to induce IL-10 in monocytes (Brady, MacDonald et al. 2003). Other viruses, such as lymphocytic choriomeningitis virus (LCMV), have been shown to induce high-levels of IL-10 by DCs and B cells upon infection (Brooks, Trifilo et al. 2006). Viruses such as Epstein-Barr virus (EBV) and other members of the family Herpesviridae are also known to encode viral homologue of IL-10 (vIL-10) within the infected host for the purpose of suppressing host immune-clearance (Slobedman, Barry et al. 2009). IL-10 has also been linked to the virulence of vector-borne protozoan parasites, such as Leishmania spps. Patients with visceral leishmaniasis demonstrate upregulated IL-10 in both spleen and serum, which has been shown to directly down-regulate Th1 cytokines such as IFN-γ, (Nylen, Maurya et al. 2007; Nylen and Sacks 2007). In animal models, improved clearance of the protozoan parasites can be achieved by the neutralization of host IL-10 production (Belkaid, Hoffmann et al. 2001; Yang, Mosser et al. 2007). Besides Leishmania spps, Toxoplasma gondii and Trypanosomal spps are other protozoans capable of eliciting host IL-10 to evade host immune clearance (Jankovic, Kullberg et al. 2007). Although many microbial pathogens can elicit host IL-10, there seemed to be a strong connection between IL-10 elicitation and persistence of vector-borne pathogens and/or long-term parasites.

**Mechanisms of IL-10 production/elicitation:** The elicitation of host IL-10 production by invading pathogens is an extremely complicated process that is not yet thoroughly understood for most models. Many cellular components that are involved in pro-
inflammatory responses are also linked to the production of IL-10. (Saraiva and O'Garra). In myeloid cells such as macrophages, IL-10 productions is initiated via signaling of surface receptors such as the TLRs (Saraiva and O'Garra). Amongst TLRs, the connections between TLR2 and IL-10 are currently the strongest and best-understood. TLR2 agonists such as peptidoglycan, lipoteichoic acid, and PAM3Cys -containing lipoprotein and lipopeptides were all shown to elicit IL-10 production both in vivo and in vitro (Chandra and Naik 2008; Moreira, El Kasmi et al. 2008; Kaji, Kiyoshima-Shibata et al. 2010). TLR2 associated co-receptors; including the scavenger receptor CD36 and the internal receptor NOD2 were also shown to be important for IL-10 production in macrophages (Chung, Liu et al. 2007; Moreira, El Kasmi et al. 2008). This suggests that pathogens expressing TLR2 agonists are more likely to elicit IL-10 from hosts. TLR9-mediated signaling is also known to elicit IL-10 production (Saraiva and O'Garra; Lenert, Goeken et al. 2003). In addition to TLRs, studies have implicated that the Fc receptors expressed on the surface of MØs can skew IL-10 production over pro-inflammatory cytokines when binding opsonized IgGs at certain densities (Bagenstose, Mentink-Kane et al. 2001; Yang, Mosser et al. 2007; Gallo, Goncalves et al. 2010).

Multiple intracellular signaling proteins and second messengers involved in IL-10 production have been identified within myeloid cells. Among the best known are those involved in the mitogen-activating protein (MAP) kinase pathways. There are three major types of MAP kinases in mammalian cells: p38, extracellular receptor kinase (ERK) and cJun-n-terminal kinase (JNK) (Dong, Xie et al. 2002). Within myeloid cells, MAP kinases were initially known to be connected through TLRs for the production of only pro-inflammatory cytokines, such as TNF and IL-1
However, recent studies have linked members of MAP kinases to the production of IL-10; in particular p38 and ERK (Saraiva and O'Garra). In both natural and non-pathogenic models, IL-10 induction in response to macrophage intake of apoptotic cells requires MAP kinase p38 (Chung, Liu et al. 2007). *Leishmania*-induced IL-10 production by the host is ERK1/2-dependent, such that specific inhibitors against ERK1/2 signaling pathway both abolished the host IL-10 production in response to *Leishmania* and improved the clearance of the pathogen from the host (Yang, Mosser et al. 2007). Several examples of viral-induced IL-10 production were also shown to be p38-dependent (Gee, Angel et al. 2007). Non-pathogenic Gram-positive *Lactobacilli spps* utilize p38 and ERK1/2 to upregulate host IL-10 while suppressing host IL-12 (Kaji, Kiyoshima-Shibata et al. 2010). Specific inhibitors against p38, such as SB203580, suppresses IL-10 production by macrophages in response to LPS while enhancing IL-12 production, implying that MAP kinase activation by TLR agonists upregulates IL-10 while down-regulating IL-12 (Chelvarajan, Popa et al. 2007).

Besides MAP kinases, other signaling cascades involved in IL-10 production include the phosphotidyl-inositol 3 kinase (PI3-kinase). This enzyme catalyzes the production of phosphotidylinositol 3,4,5 triphosphate (PI(3,4,5)P3) which in turn activates various signaling molecules through the pleckstrin (Ph) domain. A signaling molecule downstream of the PI3 kinase pathway that is associated with various cellular functions is protein kinase B or Akt (Marone, Cmiljanovic et al. 2008). In airway epithelial cells, PI3-kinase and MAP kinase p38 have both been linked to elicitation of host IL-10 by *Mycobacteria bovis* in lung epithelial cells. PI3- kinase is also involved in IL-10 production and anti-inflammatory activities in macrophages (Martin, Schifferle et
al. 2003; Polumuri, Toshchakov et al. 2007). The mammalian target of rapamycin (mTOR) or raptor, a downstream effector of PI3-kinase/Akt pathway usually known for activation of ribosomal subunit S6 in response to changes in the level of nutrients, was also linked to IL-10 production (Weichhart, Costantino et al. 2008; Saemann, Haidinger et al. 2009). Spleen tyrosine kinase (SYK), which can be involved in the activation of both the PI3 and MAP kinase cascades, is also linked to pathogen-induced IL-10 production (Yang, Mosser et al. 2007). As for putative transcription factors involved in IL-10 production, there has been evidence suggesting that Sp1 and STAT3 are involved in pathogen-induced IL-10 (Mosser and Zhang 2008). However, the role of other transcription factors including NF-κB are also implicated but are less well understood (Mosser and Zhang 2008).

**Role of IL-10 in Bb infection:** IL-10 was first linked to LD and Bb infection when isolated monocytes from primates responded to heat-killed Bb and OspA by producing high levels of IL-10 (Giambartolomei, Dennis et al. 1998). Bb infection of IL-10⁻/⁻ mice resulted in significant improvement of Bb clearance from target tissues compared to wild type mice (Brown, Zachary et al. 1999). In addition, the infectious dose 50 (ID-50) of Bb in IL-10⁻/⁻ mice was eight-fold greater than that of wt (Lazarus, Meadows et al. 2006). These findings are evidence that host IL-10 hinders the efficient clearance of Bb.

To further understand the relevance of IL-10 in LD and Bb infection, studies indicate that murine macrophages produce IL-10 in response to Bb lipoproteins through a TLR2-dependent manner (Wooten, Ma et al. 2002). Through the advent of a culture media that supports both viable Bb and healthy macrophages, studies showed that viable
Bb elicits IL-10 at greater levels than non-viable Bbs (Lazarus, Kay et al. 2008). High levels of IL-10 transcripts can be detected within 24 hours in the skin after injection of only $10^4$ viable Bb (Lazarus, Kay et al. 2008). These findings indicate that viable Bb can elicit host IL-10 production immediately upon entry into the host. The mechanism of how Bb-elicited IL-10 suppresses the immune effectors important for killing of the bacteria is just beginning to be understood. It is arguable that IL-10 production by host cells in response to Bb infection tends to exert a greater effect on the innate immune arm. Studies indicate that Bb-elicited IL-10 directly down-regulate the production of TNF and IL-6 by macrophages in response to Bb as well as make these macrophages refractory toward further stimuli (Lazarus, Kay et al. 2008). Other studies identified that Bb-elicited IL-10 activates SOC3 in macrophages to exert its anti-inflammatory functions (Dennis, Jefferson et al. 2006); this implies that multiple innate immune effectors against Bb could be suppressed. Bb-elicited IL-10 also affected the adaptive responses against Bb. Although higher levels of Bb-specific antibodies were detected in infected IL-10 $^{-/-}$ mice, the breadth of proteins recognized by these antibodies did not differ from wild-type, and passive transfer of these antibodies to SCID mice did not confer better Bb clearance compared to antibodies from infected wild-type mice (Lazarus, Meadows et al. 2006). These data suggest that the main detrimental effects of Bb-elicited IL-10 are directed against the innate immune differences that are important for controlling Bb infection. However, the details on how Bb causes a dysregulated IL-10 response and the specific immune mechanisms that are affected are poorly understood.
1-4 Significance of Thesis research:

It is clear that one strategy for Bb to establish persistence within an immune competent host is to elicit IL-10 from the host to suppress immune effectors important for Bb killing. Previous literature indicates that, in culture conditions, Bone-marrow derived macrophages (BMMs) produce IL-10 at high levels that are capable of suppressing pro-inflammatory responses. However, the principal cell types involved in IL-10 production during \textit{in vivo} Bb infection is yet to be determined. To begin this identification process, other immune cells such as cultured bone-marrow dendritic cells (BMDC) will be studied as another potential IL-10 producing cell.

As aforementioned, IL-10 produced by BMMs in response to Bb was able to suppress TNF\(\alpha\) and IL-6. However, it is not clear how Bb-elicited IL-10 affects other cellular immune mediators important for Bb clearance. \textit{In vivo} studies demonstrate that IL-12 can up-regulate IFN-\(\gamma\) by Bb-specific T cells during Bb infection (Hedrick, Olson et al. 2006). Therefore, it is important to examine whether cultured APCs produce IL-12 in response to Bb and whether IL-12 is subsequently suppressed by Bb-elicited IL-10. Chemokines such as KC have been demonstrated to be important for the recruitment of neutrophils to the site of Bb infection (Xu, Seemanapalli et al. 2007; Ritzman, Hughes-Hanks et al. 2010). Whether the production of KC and related chemokines are also affected by Bb-elicited IL-10 has yet to be identified. In addition to cytokines and chemokines, whether IL-10 downregulates other APC immune mechanisms such as phagocytosis, respiratory burst and upregulation of co-stimulatory molecules in response to Bb needs to be understood.
Very little is known regarding the mechanisms of how Bb accomplishes this host IL-10 elicitation. Unlike *Leishmania* and *Mycobacterium spps*, Bb does not appear capable of intracellular survival. Therefore, Bb must rely on extracellular interactions to elicit host IL-10. Whether or not Bb utilizes a similar secretion system to elicit the production of IL-10 from host cells as *Yersinia* and *Bordetella spps* is currently unknown. TLR2 is currently the best known host surface PRR important for Bb clearance. Although the production of IL-10 by MØs in response to Bb lipoproteins *in vitro* is TLR2-dependent (Wooten, Ma et al. 2002), whether TLR2 is also required for IL-10 production by MØs in response to viable Bb is yet unknown. The production of IL-10 by host immune cells in response to Bb occurs as quickly and more potently than most pro-inflammatory responses (Lazarus, Kay et al. 2008), thus it is possible that distinctive signaling cascades exist for these opposing pathways. Therefore, it is important to delineate the intracellular signaling pathways, such as MAP kinases and PI3-kinase, that are involved in producing IL-10 versus pro-inflammatory mediators. These findings can also facilitate the identification of putative host transcription factors utilized by Bb to rapidly turn on IL-10 production.

One challenge with *in vitro* studies is that culturing immune cells in BSK alone may inherently induce activation since BSK contains potential TLR2 agonists such as yeast extract and peptidylglycan components (Pollack, Telford et al. 1993). In order to minimize this inherent activation, a media containing 80% RPMI and 20% BSK (RPMI.B) was developed and tested for both the ability to support Bb viability while minimally activating cultured mammalian cells (Lazarus, Kay et al. 2008) (Wooten unpublished). The discovery of the modified media RPMI.B for optimal Bb and
mammalian cell culture provides a powerful tool for better assessing cellular interaction between Bb and APCs *in vitro*.

In summary, the focus of the thesis research is on three main areas: 1) Identifying APC innate immune effectors important for Bb clearance that are dysregulated by Bb-elicited IL-10, 2) Identifying the surface interactions between Bb and APCs (*i.e.*, receptors) important for Bb-elicited IL-10 and 3) Identifying intracellular signaling molecules/cascades important for Bb-elicited IL-10 production and determine if they are distinct from the production of pro-inflammatory cytokines. These findings should potentially advance the knowledge of how Bb utilizes host IL-10 to establish persistence. These findings should also aide in the design of effective therapeutics against LD that can neutralize the suppressive effects of IL-10 during Bb infection.
Chapter 2

*Borrelia burgdorferi* Elicit Dysregulated Phagocytosis and Inflammatory Responses from Murine Antigen Presenting Cells through Enhancement of IL-10 Production

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Abstract

*Borrelia burgdorferi* (Bb) is a tick-borne spirochete that is the causative agent for Lyme disease. These bacteria are notable for their ability to evade host defenses and persist extracellularly, even though infection elicits potent innate and adaptive immune responses. Our previous studies indicate that virulent Bb can potently enhance IL-10 production by antigen presenting cells (APCs), such as macrophages (MØs) and dendritic cells (DCs), and that suppression of IL-10 production significantly enhances bacterial clearance. We hypothesize that APC-produced IL-10 has autocrine activity to suppress immune responses that are important for efficient clearance of these bacteria. The goal of these studies is to delineate which APC immune functions are dysregulated by Bb-elicited IL-10 using a murine model of Lyme disease. Our studies confirmed that both APCs rapidly produce IL-10 upon exposure to *B. burgdorferi*, and that these levels inversely correlate with the production of the proinflammatory cytokines IL-6 and IL-12, as well as the chemokines KC, MIP-1α, and MIP-2. Similarly, APCs derived from IL-10⁻/⁻ mice produced significantly greater amounts of these inflammatory mediators compared to APCs from wild type mice. *In vitro* phagocytosis assays determined that most Bb are engulfed by MØs within 5 min post-infection, are trafficked into the vicinities of LAMP-1-containing compartments by 30 min, and that these trafficking activities are significantly hindered by Bb-elicited IL-10. Physiological levels of IL-10 suppress respiratory burst, but not nitric oxide production by APCs in response to Bb. Exposure to *B. burgdorferi* led to up-regulation of the surface co-stimulatory markers CD86 and MHC class II, but not CD80 and CD40; IL-10 had little effect on these expression patterns. Overall, our data indicates that IL-10 elicited from both MØs and DCs results in
decreased production of critical pro-inflammatory mediators and suppress phagocytosis events that are important for mediating both innate and adaptive immune responses to Bb.
Introduction

Lyme disease is a re-emerging infection caused by the tick-borne spirochetal bacterium *Borrelia burgdorferi* (Bb), an organism which may persist within various host tissues for a long period of time (Burgdorfer, Barbour et al. 1982; Steere, Coburn et al. 2004). The mechanism of how Bb persists is not clearly known. However, Bb uses many strategies to evade host immune system (Tilly, Rosa et al. 2008). Bb does not exhibit complete immune evasion as primary infection elicits a strong host inflammatory response as characterized by erythema migrans (Dandache and Nadelman 2008). However, while symptoms initially abate, the pathogen is not completely cleared and late manifestation of the disease can occur over time.

T cells, particularly those involved in cell-mediated responses, play very little role in the immune clearance of Bb (Barthold, Sidman et al. 1992; de Souza, Smith et al. 1993; Brown and Reiner 1999). Humoral immunity does play some role in protection against Bb infection, as passive transfer of immune sera from infected animals to naïve animals prevents disease; however, if given post-infection, it has no effect on disease progression or severity (Barthold and Bockenstedt 1993). Bb carries out antigenic variation, constantly switching the expression of certain surface antigens such as lipoproteins VlsE (Singh and Girschick 2004; Fikrig and Narasimhan 2006). Besides VlsE, many other Bb surface lipoproteins appear to promote host immune evasion. These include the Erp/CRASP members which are also involved in antigen variation as well as the sequestering of key inhibitors of the complement pathway, such as factor H (Kraiczy, Skerka et al. 2001; Woodman, Cooley et al. 2007). In addition, lipoprotein OspB can attenuate neutrophil activation (Hartiala, Hytonen et al. 2008). Overall, these
findings indicate that Bb possesses many immune-evasive strategies against host immune effectors, and helps promote persistence in immune-competent hosts for long periods of time.

Certain members of the toll-like receptor (TLR) family, and particularly TLR2, are important in Bb clearance (Lien, Sellati et al. 1999; Wooten, Ma et al. 2002; Wooten, Ma et al. 2002). TLR2 deficient mice have been shown to have a much greater pathogen burden and lower infection dose 50 (ID$_{50}$) than wild type (wt) mice (Wooten, Ma et al. 2002). In addition, TLR2 expressed on phagocytes, such as macrophages, reacts strongly to surface lipoproteins of Bb (Hirschfeld, Kirschning et al. 1999; Wooten, Ma et al. 2002). MyD88, the intracellular adaptor protein that is associated with TLR2 plays a critical role in phagocytosis of Bb as well as the induction of pro-inflammatory cytokines by macrophages in vitro (Bolz, Sundsbak et al. 2004; Liu, Montgomery et al. 2004; Shin, Isberg et al. 2008). In summary, TLR2 and innate immunity are important for Bb clearance.

The anti-inflammatory cytokine IL-10 (Moore, de Waal Malefyt et al. 2001; Couper, Blount et al. 2008) is known to play a significant role in the immune clearance of Bb. Deficiencies in IL-10 often lead to uncontrolled inflammatory conditions, such as colitis and autoimmune diseases (Moore, de Waal Malefyt et al. 2001). In the case of Lyme disease, although IL-10 deficient mice (IL-10$^{-/-}$) showed greater levels of pathology, they also display reduced levels of Bb loads in tissues such as the ankle, and the ID$_{50}$ of Bb was about eight-fold higher than wt mice (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006).
Although IL-10 can exert its activity across both the innate and the adaptive immune systems, the preliminary data suggest that, in the case of Bb infection, IL-10 may play a more important role by suppressing innate immunity. These include data showing high levels of IL-10 detected in host skin tissue near the injection site within 24 hours of infection (Lazarus, Kay et al. 2008), which suggests it is most likely produced by resident antigen presenting cells (APCs) such as macrophage and dendritic cells. Data generated from in vitro studies indicated that macrophages elicit abnormally high levels of IL-10 when exposed to Bb lipoproteins (Brown, Zachary et al. 1999). Similar assays have shown that live intact Bb grown in optimal culture conditions elicit the highest levels of IL-10 from macrophages as compared to dead Bb or those cultured in sub-optimal conditions (Lazarus, Kay et al. 2008). Kinetic studies indicate that the rate of IL-10 production by macrophages stimulated with live Bb is extremely rapid, where detectable levels are seen within four hours post-stimulation (Lazarus, Meadows et al. 2006). Furthermore, the IL-10 elicited by Bb can subsequently down-regulate pro-inflammatory cytokine production, such as TNF-α and IL-6, by the infected macrophages. Supernatants from Bb-infected macrophages are capable of suppressing naïve macrophages from subsequent stimulation by LPS and this effect can be reversed by IL-10 neutralizing antibodies (Lazarus, Kay et al. 2008). These results argue that Bb-elicited IL-10 down-regulates activation of immune effectors by APCs and thus could be an efficient way for the pathogen to evade immune clearance and to persist in vertebrate hosts.

We hypothesize that Bb-elicited IL-10 production by APCs subsequently suppresses various immune mediators important for the killing of Bb. Our goal is to
examine whether both macrophages and dendritic cells produce IL-10 in response to Bb-stimulation and whether APC immune mediators such as pro-inflammatory cytokine/chemokine production, initiation of phagocytosis, production of respiratory burst, and the upregulation of surface MHC II/co-stimulatory molecules are suppressed by Bb-elicited IL-10. These *in vitro* analyses will be performed using a defined medium that keeps Bb alive for over 24 hours without activating APCs. The findings of our study should allow better understanding of how Bb utilizes the dysregulated host IL-10 production to evade immune clearance.

**Material and Methods**

*B. burgdorferi growth and viability:* Clonal N40 isolate of Bb *sensu stricto* was generously provided by Steve Barthold (University of California, Davis, CA) (Barthold, Beck et al. 1990). These bacteria are maintained in Barbour Stoenner and Kelly media II (BSK-II). Clonal B31 isolate of Bb expressing green fluorescent protein (GFP) on an Erp promoter was kindly provided by Brian Stevenson (University of Kentucky, Lexington, KY) (Miller, von Lackum et al. 2006) and are maintained in BSK-II + 2% kanamycin (BSK²). All *in vitro* experiments were performed using bacteria that are between passages 5-7 *ex vivo*.

**Animal usage:** C57BL/6 wild-type and IL-10⁻/⁻ mice were obtained from Charles River Laboratories (NCI-Frederick). All animals were housed in the Department of Lab Animal Research at the University of Toledo Health Science Campus according to
National Institute of Health guidelines for the care and use of laboratory animals. All usages protocols were reviewed and approved by the Institutional Animal Care and Usage Committee.

Expansion of bone-marrow derived APCs: For bone-marrow derived macrophages (BMM) we used procedures described previously (Lazarus, Meadows et al. 2006). Briefly, dissociated marrow tissues from the limb bones of C57BL/6 (wt) or IL-10^{-/-} mice were isolated and cultured for 6 days in RPMI media containing 30% L929 cell supernatants. At the end of the culture period, the adherent cells were collected and re-seeded onto tissue culture plates in culture media composed of RPMI+10%FBS supplemented with 20% BSK-II (RPMI.B) for at least 8 hours prior to in-vitro assays. For bone-marrow derived dendritic cells (BMDC), we use RPMI media containing 10ng/ml recombinant GM-CSF (R&D, Minneapolis, MN) for 5 days. At that time the non-adherent cells are harvested and plated in RPMI.B for at least 18 hours prior to experiments. The phenotype of these expanded bone marrow cells was examined using fluorescent antibodies against selected surface markers described below (BD Pharmingen, San Diego, CA) and subsequently detected using a flow cytometer (FACS Caliber, Becton Dickenson, San Diego, CA). For naïve BMMs, we verified that these cells are high in CD11b and F4/80 (greater than 80% positive), while low on MHC class II and CD11c (less than 5% positive). Alternatively, naïve BMDCs were high in CD11b, CD11c and MHC class II (Figure 1).
In vitro co-culture of Bb with bone-marrow APCs: For co-culture experiments, naïve APCs were seeded on 24-well tissue-culture treated plates at a concentration of 2-3x10^5 /ml. Live Bbs were added to each well at a multiplicity of infection (MOI) of 10. After adding bacteria, the culture plates were centrifuged at 300xg for five minutes to facilitate contact between Bb and APC. These plates were then incubated in 37°C for the desired times. All experimental conditions were performed in triplicate wells.

Detection of secreted cytokines: Supernatants from cultured APCs were collected at various times post-infection. A sandwich ELISA using paired antibodies was used to detect secreted cytokines as described previously (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). Briefly, cell supernatants were incubated overnight on EIA high-binding plates (Costar) pre-coated with monoclonal antibodies against the cytokine of interest (e.g. IL-10). Bound cytokines were visualized using biotinylated detection antibodies and avidin-HRP. Concentration of cytokine levels were determined by measuring the absorbance at a wavelength of 490 nM using a Versamax® (Molecular Devices) 96-well plate reader and comparing to appropriate cytokine standards. All antibodies (purified mAbs and biotinylated Abs) and recombinant cytokine standards were purchased from BD Biosciences and avidin-HRP was purchased from Vector Laboratories (Burlingame, CA).

Detection of RNA transcripts: Parallel in vitro co-culture experiments using BMM and BMDC were performed as described above, except total RNA was harvested from the pooled triplicate wells using an RNeasy® kit (Qiagen, Valencia, CA). Similar
experiments were performed at least three times. Total RNA was reverse transcribed into cDNA using ImProm II® reverse transcriptase (Promega, Madison, WI) per the manufacturer’s instructions. This cDNA was then quantified using real-time quantitative PCR techniques with the Light Cycler (Roche) rapid fluorescence temperature cycler, as previously described (Lazarus, Meadows et al. 2006). The primers used for the PCR were designed in house and purchased through Integrated DNA Technologies (Coralville, IA) (see list in Table 1).

**Immunofluorescence microscopy (IM) imaging and phagocytosis assay:** For assays involving imaging of Bb phagocytosis, APCs were seeded in 12-well plates containing glass cover slips within each well at a concentration of 1.5-2x10^5 cells per well in RPMI.B. For BMDCs, glass cover slips were coated with 0.01% poly-L-lysine (Sigma) for at least 24 hours prior to cell seeding to enhance cell adherence. Seeded APCs were either left untreated or were pre-treated overnight with 10 MOI of live N40 Bb or recombinant IL-10 at 2ng/ml before performing analysis (BD Pharmingen). For the phagocytosis assay, GFP-producing B31 Bb were added to each well at a MOI of 10 and centrifuged at 300xg for three minutes to facilitate Bb-BMM contact. At the indicated times post-infection, supernatants were removed and the cells washed two times in PBS to remove unbound Bb. APCs were then fixed in 4% paraformaldehyde overnight prior to immunochemical staining. To identify lysosomal compartments, fixed APCs were first membrane-permeablized with 1% Triton-X-100 and then stained with 2 µg/ml of the ID4B (Developemental Hybridoma, University of Iowa, Iowa City, IA), antibody specific
for lysosome-associated membrane protein-1 (LAMP-1) and visualized with 2 µg/ml TRITC-labeled secondary goat anti-rat antibody (Southern Biotech, Birmingham, AL). DAPI (Fisher Scientific) at 250nM was used to visualize the nuclei. Cover slips containing the stained APCs were mounted onto glass slides using Fluoromount® (Southern Biotech). The mounted cover slips were then examined using a phase and immunofluorescence microscope (Leica). Epifluorescent images of fields containing 75-150 APCs were captured at 200x magnification and overlayed on bright-field images using Q Capture® software. For each slide, at least three separate fields were assessed. For quantitative analysis of phagocytosis, the percentage of APCs containing at least one internalized Bb particle was determined for each field as the number of APCs containing Bb / total number of APCs per field. This ratio was averaged for the total fields per three independent experiments.

**Determination of reactive oxygen intermediates (ROI):** Respiratory burst was determined using a 2′, 7′-dichlorodihydrofluorescein-diacetate probe (DCFH-DA) (Molecular Probes) which fluoresces in the presence of intracellular ROIs (DeLeo, Allen et al. 1999; Deng, Xu et al. 2008). Intracellular ROI converts DCFH-DA into DCF, where its fluorescent intensity is detected at 480nm excitation/520nm emission. APCs were seeded in 96-well black plates with clear bottom (Costar) at 1x10^5 cells per well in RPMI.B for 10 to 15 hours prior to Bb-stimulation. Immediately preceding stimulation, the RPMI.B media was washed off and APCs were pre-loaded with 10µM DCFH-DA in serum-free HBSS for 15-20 minutes at 37°C before removing the excess dye. To allow metabolism of the internalized DCFH dye, cells were incubated for an additional 15
minutes in fresh HBSS at 37°C prior to stimulation with viable Bb. During this time, some of these loaded APCs were treated with recombinant IL-10 at physiological levels (~2ng/10^5 cells). Upon Bb stimulation, the cells were centrifuged at 300xg for three minutes to facilitate Bb-APC contact. The relative fluorescent intensity unit (RFU) and the change in RFU over time in each well was acquired using a fluorimeter (FluoStar®, BMG) at 1 minute intervals for 45 minutes.

**Determination of reactive nitrogen intermediates (RNI):** To detect nitric oxide (NO) production, supernatants from APCs stimulated with viable Bb in the presence/absence of anti-IL-10 antibodies were collected and Griess method was performed to determine nitrite (NO_2) levels as described previously (Ding, Nathan et al. 1988). Briefly, equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% N-(1-napthyl) ethylenediamine dihydrochloride in 60% acetic acid were mixed with 50 µl of cell supernatants in a 96-well plate. Concentrations of NO_2 in the supernatant were determined based on the absorbance values acquired at 570 nm wavelengths using the Versamax® 96-well plate reader (Molecular Devices) and compared to set concentrations of NaNO_2 standard.

**Detection of surface co-stimulatory molecules:** Expression of surface co-stimulatory molecules was assessed by flow cytometry. APCs were seeded onto six-well plates at a concentration of 1 x10^6 cells/ml and allowed to adhere overnight before adding Bb at MOI of 10. After overnight incubation, cells are washed and ind in ice-cold PBS for 30 minutes and recovered by gentle scraping. The recovered cells were transferred to
polystyrene tubes and incubated for 30 minutes with blocking buffer (PBS containing 1% BSA) along with 10 µg/ml anti-Fc receptor antibodies (BD Pharmingen) on ice. Next, APCs were incubated with either FITC or PE-conjugated monoclonal antibodies against cell surface markers CD11b, CD11c, MHC II, CD80, CD86, CD40 and F4/80 (BD Pharmingen) all at 5 µg/ml. After 20 minutes incubation on ice, the cells were washed three times with fresh PBS to remove excess antibodies. Next, the antibody-labeled APCs were loaded with 0.02% propidium iodide (Sigma) and levels of fluorescent signals were measured using the FACS Caliber® flow cytometer. Quantitative analysis of the flow cytometry data (e.g. mean fluorescent intensity and percent positive) was performed using Cellquest® software.

**Statistical analysis:** The statistical significance of the quantitative differences between the different sample groups was determined by application of Student’s T test (two-tailed, two sample equal variance). P values of <0.05 were considered to be statistically significant.

**Results**

**Viable Bb elicit high levels of IL-10 production by both BMMs and BMDCs in vitro:** Kinetic studies were performed to assess the production of IL-10 by APCs and the effect on production of pro-inflammatory cytokines. The addition of Bb to wt BMMs and BMDCs elicited significant levels of secreted IL-10 within eight hours of simulation, as determined by ELISA (Figure 2, top panels). Viable Bb also elicited a variety of other
pro-inflammatory cytokines by both BMMs and BMDCs (figure 2, middle/bottom panel), including IL-6, IL-12, and TNFα (data not shown), with similar kinetics to IL-10. To test whether Bb elicited IL-10 suppresses the production of pro-inflammatory cytokines from APCs, parallel experiments were performed with BMM and BMDCs isolated from IL-10 −/− mice. As expected, IL-10 −/− APCs do not produce IL-10 in response to Bb, however, the production of all assessed proinflammatory cytokines were higher than that produced by wt APCs. Parallel experiments were performed using wt APCs stimulated with Bb in the presence of neutralizing antibodies against IL-10 (α-IL-10) which also showed a similar increase in the production of pro-inflammatory cytokines when the Bb-elicited IL-10 was neutralized. These findings suggest that Bb-elicited IL-10 can act back on BMMs and BMDCs to suppress the production of pro-inflammatory cytokines that are important for mediating inflammatory activity by Bb.

**Chemokine production by APCs is suppressed by Bb-elicited IL-10:** Neutrophil-recruiting chemokines such as KC or CXCL1 have been demonstrated to play critical roles during Bb infection (Montgomery, Lusitani et al. 2004; Xu, Seemanapalli et al. 2007). Since KC and other similar chemokines are produced by resident APCs following stimulation (De Filippo, Henderson et al. 2008), we assessed whether Bb-elicited IL-10 can down-regulate chemokine production by APCs. Addition of Bb induced up-regulation of the transcript levels of many chemokines, including KC, MIP-2, MIP-1α, by BMMs and BMDCs (Figure 3A) as early as 2-4 hours post-infection; RANTES production was constitutively high and was not enhanced after Bb addition. Similar to the pro-inflammatory cytokines, chemokine upregulation is also further enhanced in the
presence of α-IL-10 (Figure 3A and B), with significant differences observed by 8-12 h post-stimulation. Experiments performed using BMDCs demonstrated similar trends of chemokine enhancement in the absence of secreted IL-10 as seen in BMMs. To further validate our findings, parallel experiments were performed using APCs derived from IL-10⁻/⁻ mice and these data also showed that chemokine transcript levels were substantially elevated in the absence of Bb elicited IL-10 (data not shown).

IL-10 hinders Bb uptake and intracellular trafficking of Bb by BMMs: To assess whether Bb-elicited IL-10 directly affects phagocytosis of the bacteria by the BMMs, an assay system was developed where images of BMMs infected with GFP-expressing B31 strain of Bb were captured and analyzed at different times post-infection by immunofluorescent microscopy (IM). Bb intake by BMMs occurred as early as five minutes post-infection, as demonstrated by the presence of internalized Bb particles within the APCs (Figure 4A). At 15 and 30 minutes post-infection, the number of bacterial particles (green) that were internalized by APCs increased. Upon internalization, almost all Bb lost their linear morphology and appeared as tight circles. Previous literature suggests that phagocytosed Bb is trafficked to LAMP-1 containing compartments within macrophages (Liu, Montgomery et al. 2004; Shin, Isberg et al. 2008). Therefore, we stained LAMP-1 with a contrasting color (red) against the green Bb particles in order to better track the internalized Bbs. To delineate whether the images shown in Figure 4A truly demonstrated Bb internalization, parallel BMM cultures were pre-treated with 2μM cytochalasin D, an inhibitor of actin polymerization, for 45 minutes.
prior to Bb infection. These BMMs demonstrated an inability to take up Bb and all Bb maintained their linear morphologies (data not shown).

To determine whether IL-10 affects the uptake and trafficking of Bb by BMMs, the percentages of BMMs containing internalized, non-linear Bb particles per four field of view (e.g. at least 100 BMMs) were compared between WT BMMs and those pretreated with 4ng/ml recombinant IL-10. At 30 minutes post-infection, BMMs pre-treated with rIL-10 demonstrated significant reduction in percent Bb intake as compared to control BMMs (Figure 4B). To provide a more physiological model, IL-10 was induced naturally by pre-infecting BMMs with non-fluorescent N40 Bbs overnight before re-infecting the same BMMs with the fluorescent GFP-B31 strain. This produced a similar reduction of percent Bb intake with the treatment with recombinant IL-10 (Fig 4B). To delineate whether the suppression of BMM phagocytosis by the “re-infection group” is due to IL-10, neutralizing α-IL-10 were added during the pretreatment with N40 Bb and this reverts the percent Bb uptake back to the untreated Bb levels (Fig 4B). Similar assays were performed to determine the effects of Bb-elicited IL-10 on Bb phagocytosis by BMDC. Wild type BMDC displayed a similar patter and rate of Bb uptake as seen in BMMs. However, unlike the BMMs, the presence of rIL-10 or Bb-elicited IL-10 did not affect Bb uptake and trafficking by BMDC.

**Bb-elicited IL-10 suppresses the production of reactive oxygen intermediates (ROI) but not reactive nitrogen intermediates (RNI) in response to Bb:** Live Bb and Bb components are capable of eliciting ROIs in phagocytic cells such as macrophages and neutrophils (Morrison, Weis et al. 1997; Wooten, Ma et al. 2002; Marangoni, Accardo et
al. 2006). To test whether Bb-elicited ROI production in BMMs is affected by IL-10, a dichlorodihydrofluorescein (DCFH) assay was employed to detect intracellular ROI levels in BMMs in response to Bb. Stimulation of BMMs with Bb caused rapid upregulation as indicated by both the raw RFU units as well as the slope of change (Figure 5A, top and bottom). When a physiological amount of IL-10 (1-2ng/10^5 cells) was added to BMMs prior to Bb stimulation, the level of Bb-induced ROI production dropped significantly. Similar experiments were performed using BMDCs and the results indicated that BMDCs produce very little detectable ROI compared to BMM (Figure 5B, top and bottom panel). To ensure that the RFU values obtained reflects internal ROS generation by APCs, parallel experiment was performed where APCs were stimulated by Bb in the presence of NADPH oxidase inhibitor diphenylene iodonium (DPI) at 10 µM. Results in Figure 5 indicated that DPI-treated cells showed no increases in RFU values. Next, RNI production by both BMMs and BMDCs in response to viable Bb was assessed using the Griess assay which involved the measurement of nitrite as a more stable but quantifiable byproduct of nitric oxide in culture supernatants. The addition of Bb caused a significant upregulation of nitric oxide and parallel wells stimulated in the presence of an IL-10 blocking antibody showed a similar upregulation of nitric oxide, suggesting that Bb-elicited IL-10 had no effect on nitric oxide production (Figure 6). Alternatively, the addition of Bb to BMDC in the presence or absence of rIL-10 had little effect on nitric oxide production, suggesting that Bb does not elicit nitric oxide from BMDCs.

Viable Bb up-regulates a subset of co-stimulatory molecules on the surface of APCs:
Cells of the adaptive immune system such as CD4 and CD8 T cells appear to play a very
limited role in Bb immunity. One explanation for this lack of response could be that Bb interactions with APCs depress surface expression of co-stimulatory molecules on their surface, preventing appropriate T cell responses. To address this, bone-marrow APCs isolated from both WT and IL-10−/− mice were stimulated with live Bb for 24 hr before visualizing the levels of critical co-stimulatory markers by FACS analysis. Bb stimulation resulted in a significant upregulation of CD86 by both BMM and BMDCs, but these levels were further enhanced in IL-10−/− BMM and BMDCs (Figure 7), suggesting that Bb-elicited IL-10 suppresses CD86 expression. MHC class II molecules were also upregulated on BMDCs by Bb stimulation, but not on BMM, and these levels were unaffected by IL-10. Interestingly, the inherent levels of all these surface molecules present on the surface of the APCs were already high. This could explain why neither CD40 nor CD80 appear to be upregulated in response to Bb by either BMM or BMDC (Figure 7 A and B). To further verify this, parallel experiments using Salmonella typhimurium LPS and/or gamma-interferon, which are well-known for their abilities to upregulate CD40 and CD80 on APCs, showed only a slight upregulation of these co-stimulatory markers (Figure 7C). However, despite the inherent levels of the surface markers, the expression of CD86 on the surface of APCs was still upregulated in response to Bb and that this upregulation of CD86 is further enhanced in the absence of IL-10.

**Discussion**

Bb is an obligate parasite whose natural infection cycle requires it to persist in an immunocompetent host for an extended time post-infection. Host IL-10 produced in
response to Bb infection can suppress the immune-clearance of these bacteria in vivo (Lazarus, Meadows et al. 2006). Further, resident APCs such as macrophages may be the primary producers of IL-10 in response to Bb infection, and the resulting IL-10 may subsequently down-regulate the production of pro-inflammatory cytokines such as TNF-α by macrophages (Lazarus, Kay et al. 2008). The goal of this study was to determine the role of macrophages and dendritic cells as a source of Bb-elicited IL-10, and to determine whether any of their critical immune functions are suppressed by these physiological IL-10 levels. Like macrophages, dendritic cells are also very instrumental in host defense against certain pathogens, particularly those known to infect skin and mucosal epithelial cells (Banchereau, Briere et al. 2000). There have been several studies reporting that interactions between dendritic cells and spirochetes in vitro can lead to elicitation of pro-inflammatory activities and maturation of the dendritic cells (Bouis, Popova et al. 2001; Suhonen, Komi et al. 2003; Miller, Ma et al. 2008). Our findings indicate that BMDCs produce equivalent (if not greater) IL-10 levels in response to live Bb as BMMs under similar conditions (Figure 2). BMDCs also produce high levels of pro-inflammatory cytokines such as IL-6 and IL-12 in response to live Bb at similar levels as BMMs. Furthermore, this pro-inflammatory cytokine response by BMDCs is significantly suppressed by Bb-elicited IL-10. These findings further validate resident APCs being the primary producers and targets of Bb-elicited IL-10, thus supporting the hypothesis for this study. The fact that multiple cell types produce IL-10 in response to Bb further illustrates the importance of host IL-10 elicitation as a virulence factor for evading immune clearance. Although resident APCs are thought to be primary producers of host IL-10 during innate immune responses, other hematopoietic cells as well as non-
hematopoietic cells are also capable of producing IL-10 when stimulated with various agonists (Moore, de Waal Malefyt et al. 2001; Mendez-Samperio, Trejo et al. 2008). Since Bb interacts with multiple tissues and cell types, further experiments are required to delineate other host cells that may be major producer of or responders to Bb-elicited IL-10 during Bb-infection in vivo.

A number of different chemokines that recruit leukocyte to infection sites are upregulated during Bb infection (Behera, Hildebrand et al. 2006). In these studies, multiple chemokines were also shown to be upregulated by both BMM and BMDCs in response to Bb. In the absence of Bb-elicited IL-10, these chemokine levels were substantially enhanced, and particularly, the neutrophil-recruiting chemokines, such as KC (CXCL1) and MIP-2 (CXCL2), suggesting that recruitment and activation of neutrophils to the infection site could be hindered by Bb-elicited IL-10. Previous studies have shown a clear connection between KC-mediated neutrophil recruitment to Bb infection site and immune clearance of Bb (Xu, Seemanapalli et al. 2007). In addition, IL-10 suppresses many neutrophil activities in vitro in response to different stimuli (Marangoni, Accardo et al. 2006; Rossato, Cencig et al. 2007). Together, these findings suggest that IL-10 effects on suppressing chemokine-mediated recruitment of neutrophils may play a major role in allowing the early escape of Bb from immune clearance. We predict that infection studies comparing skin histopathology between wt and IL-10⁻/⁻ mice will reveal the significance of these effects.

Besides latent pro-inflammatory mediators (i.e. those that require gene expression), IL-10 is also known to affect immediate APC activities such as phagocytosis and respiratory burst. In our phagocytosis study, the microscopy images were consistent
with other published studies, in that Bb was taken up by BMMs as early as five minutes post-infection in unextended form, and were localized to the vicinity of LAMP-1 containing compartment as early as 30 minutes after intake (Montgomery and Malawista 1996; Liu, Montgomery et al. 2004; Shin, Isberg et al. 2008; Salazar, Duhnam-Ems et al. 2009). Although whether or not Bb has precisely trafficked into the lumen of phagolysosomes was not assessed, the fluorescent images clearly distinguish internalized Bb within APCs from those that remained extracellular. We addressed the effects of Bb-elicited IL-10 on phagocytosis in two ways: 1) pretreatment with physiologic doses of rIL-10 and 2) Pretreatment with non-fluorescent Bb to elicit natural production of IL-10. In each case, the trafficking of Bb was significantly suppressed by 30 minute post-infection, and the suppression was lost in the presence of IL-10 blocking antibodies, confirming that the effects were IL-10 specific. Thus, Bb-elicited IL-10 appears to have a direct effect on phagocytosis and trafficking Bb, and presumably affects the killing efficiency during clearance. Further studies would be needed to delineate which phagocytosis aspects are affected by IL-10, such as F-actin polymerization resulting in the formation of the phagocytic cup in BMMs. The protein Arp2/3 and Rac activation are involved in Bb-phagocytosis by BMMs (Shin, Miller et al. 2009). Therefore, it would be interesting to test whether the activities of these components are a target of Bb-elicited IL-10. Alternatively, uptake and clearance by BMDCs appeared to be unaffected by IL-10. This could be a reflection of the relatively low phagocytosis rate exhibited by BMDCs compared to BMM, but could also reflect a general mechanistic difference between BMMs and BMDCs in terms of Bb retention and killing.
The production of various ROIs such as superoxide (O$_2$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH'), during respiratory burst within phagocytes are essential to the killing of many bacterial and fungal pathogens. Patients with chronic granulomatous disease (CGD), a disorder in which the superoxide-generating enzyme NADPH oxidase is non-functional, are highly susceptible to infectious diseases (Heyworth, Cross et al. 2003). Although there are currently no published reports demonstrating the role of host ROIs in Bb clearance, ROIs can damage various Bb components, particularly that of Bb lipoproteins (Boylan and Gherardini 2008; Boylan, Lawrence et al. 2008). In addition, the expression of superoxide dismutase (SOD), which rapidly converts the highly reactive superoxide to the less reactive hydrogen peroxide, is required for Bb survival in the host (Esteve-Gassent, Elliott et al. 2009). Our finding that Bb-elicited ROI is suppressible by IL-10 clearly suggests how IL-10 might protect Bb from killing by the ROI produced by host immune cells. The mechanisms for IL-10 suppression of ROI production in phagocytes is beginning to be understood, as IL-10 can directly interfere with the assembly of the NADPH oxidase enzyme complex (Dang, Elbim et al. 2006; Qian, Hong et al. 2006). These methods are challenging for assessing Bb \textit{in vitro}, since DCF assays cannot be performed in media that contain sera, due to interference with fluorescence readout. Thus, we could not perform pre-stimulation assays to naturally produce IL-10, but instead had to pre-treat with physiological levels of r IL-10. Nevertheless, we observed that these levels of IL-10 suppress ROI production by BMMs in response to Bb, thus providing another possible mechanism for escaping clearance by macrophages.
The production of reactive nitrogen species, such as nitric oxide (NO), by phagocytes and APCs is also instrumental in the killing of many pathogens (Nathan and Hibbs 1991). Although BMMs produced NO in response to Bb infection, these levels were not suppressed by IL-10, as indicated both by Griess analyses and iNOS transcript levels (Figure 6 and data not shown). While Bb is susceptible to killing by NO, complete suppression of these pathways do not appear to have a major effect on Bb clearance *in vivo* (Seiler, Vavrin et al. 1995; Brown and Reiner 1999). Due to the fact that NO generation by macrophages occurs within the cell, the effect of NO in pathogen killing has been mainly studied in bacteria capable of intracellular invasion (Myers, Tsang et al. 2003). Interestingly, as with Bb, the elicitation of NO by intracellular pathogens such as *Paracoccidioides brasiliensis* from macrophages is also independent of IL-10. However, IL-10 did suppress the enhanced levels of the pathogen-induced NO production seen in macrophages that were pre-treated with interferon gamma or TNFα (Moreira, Dias-Melicio et al. 2010). Therefore, although the direct effect of IL-10 on NO production in by APCs response to Bb was not observed, it is possible that IL-10 can still exert indirect effects on the production of Bb-elicited NO by APCs, via suppressing the activities of certain NO-enhancing cytokines (*e.g.* interferon gamma).

APCs play a major role in bridging the innate and adaptive immune responses by the presentation of antigens on MHC class II molecules and the surface expression of co-stimulatory molecules, such as CD40 and the B7 family molecules CD80 and CD86. *In-vitro* stimulation by both intact bacteria, as well as bacterial components (*e.g.* agonists), upregulate MHC and these co-stimulatory molecules on APCs (Segura, Su et al. 2007; Loof, Goldmann et al. 2008). However, when APCs were stimulated with viable Bb,
only CD86 and MHC class II were up-regulated on the cell surface, with no changes seen in CD40 or CD80 levels. Although BMDCs were thought to be the primary cells expressing these co-stimulatory molecules, similar patterns (yet lower MFI values) were seen in BMMs stimulated with Bb, with the exception of MHCII. A robust up-regulation of CD86, but only a slight upregulation of CD80 or CD40 in APCs stimulated with live Bb has been previously reported (Suhonen, Komi et al. 2003). Others have shown in Bb-infected animals that injecting blocking antibodies against CD86, to prevent its interaction with its T cell ligand, CD28, resulted in lower amounts of Bb-specific antibodies and a skew toward Th1 responses; whereas blocking CD80 failed to show any significant changes unless combined with the blocking of CD86 (Shanafelt, Kang et al. 1998). These studies suggest that CD86 may be a critical co-stimulatory molecule for generating adaptive immunity against Bb, and CD86 suppression by Bb-elicited IL-10 may critically affect the adaptive response to Bb infection. Long-term host parasites, like the nematode *Heligmosomoides polygyrus*, evade adaptive immune response by secreting molecules that suppress the ability of dendritic cells to activate specific T cells against the pathogen (Segura, Su et al. 2007). It is possible that Bb is using a similar strategy to suppress APC activity, but through the dysregulated production of host IL-10. Studies to assess how Bb-stimulated APCs prime naïve T cells during infection may provide insight as to the importance of these interactions in the development of Lyme disease.

The primary objective for this study was to report the effects of Bb-elicited IL-10 on the APC immune responses that are most likely to be involved in Bb clearance. Our findings suggest that these IL-10 levels can effectively suppress multiple pathways involved in phagocytosis of Bb, as well as production of multiple inflammatory
mediators, in both macrophages and dendritic cells responding to Bb in vitro. Additionally, we have confirmed that Bb interactions with these APCs can depress surface expression of key co-stimulatory surface receptors and that Bb-elicited IL-10 can also downregulate the expression of another co-stimulatory molecule, CD86 that appears important for adaptive responses to Bb infection. Together, these findings support previous works suggesting that Bb-elicitation of IL-10 is an important mechanism for dysregulating host immune responses against this pathogen, thus helping Bb to evade immune clearance and establish persistent infection.
**Table I. List of PCR primers used**

<table>
<thead>
<tr>
<th>Name of primer (mouse)</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>CXCL1 (KC)</td>
<td>5'-GGGATTCACCTGAAGAACATCAGG-3'</td>
<td>5'-TTTCTGACCAAGGGAGCCTCAC-3'</td>
</tr>
<tr>
<td>CXCL2 (MIP-2)</td>
<td>5'-AGTGAATCGCCTGCAATG-3'</td>
<td>5'-GAGAGTGCTATGACTTCCTGCTG-3'</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>5'-CGGAAATCCGGAGACTCTGAGGCACTTCAATG-3'</td>
<td>5'-CGGATCCGGACTGAAAGTCCTCCACCACTGC-3'</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>5'-GCCCCACGTGAGAATATTCTCCTAC-3'</td>
<td>5'-CTTGAACCCACTTCTACTCTGTG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-TGGAATCTGTGGCATCCATGAAA-3'</td>
<td>5'-TAAAACGCGTCGTAACATCCG-3'</td>
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Figure 1

**BMMS**

<table>
<thead>
<tr>
<th></th>
<th>percent positive</th>
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<tbody>
<tr>
<td>CD11b</td>
<td></td>
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<tr>
<td>CD11c</td>
<td></td>
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<tr>
<td>F4/80</td>
<td></td>
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<td>MHC II</td>
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**BMDC**

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<tr>
<td>CD11b</td>
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<tr>
<td>CD11c</td>
<td></td>
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<td>MHC II</td>
<td></td>
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</tbody>
</table>
Figure 1. FACS analysis of surface markers on naïve BMMs and BMDCs. Bone marrow-derived naïve MØs and DCs were collected prior to addition of Bb, stained with antibodies specific for the indicated surface markers, and analyzed by flow cytometry.
Figure 2. Effects of Bb-elicited IL-10 on the production of proinflammatory cytokines by BMM and BMDC in vitro. BMMs or BMDC from either wild type (wt) C57B/6 or IL-10$^{-/-}$ mice cultured in RPMI.B were stimulated with viable N40 Bb at an MOI of 10 at 37°C. Culture supernatants were collected at the indicated times post-stimulation with Bb and cytokine content assessed by sandwich ELISA. Red circles represent values obtained from wt cells, whereas blue circles represent those from IL-10$^{-/-}$ mice. Each data point represents the average of triplicate samples and is representative of at least three separate experiments. Open circle represents unstimulated control where as closed circle represents Bb-stimulated cells. Statistically significant (P<0.05) values are indicated between Bb-stimulated versus unstimulated APCs (*), or stimulated wt versus IL-10$^{-/-}$ APCs (**).
Figure 3

A

![Graphs showing cytokine expression](image)
Figure 3. Effects of Bb-elicited IL-10 on chemokine expression by BMM and BMDC: APCs from C57BL/6 wt mice cultured in RPMI. B at 37°C were stimulated with viable Bb at 10 MOI. At the time of Bb stimulation, cultured APCs were treated with either a monoclonal antibody which neutralizes the effects of secreted IL-10 (anti-IL-10 mAb; 1µg/ml) or a matching isotype IgG2a control antibody (isotype mAb). At indicated times post-infection, APCs were lysed and the total RNA from the cell lysates was purified. Total RNA was reverse transcribed into cDNA where quantitative PCR was performed using the primers listed in Table 1. The transcript levels of each chemokines are normalized to the transcript levels of beta actin. (A). Relative transcript levels of specific chemokines at indicated time points expressed as fold-induced over baseline (unstimulated APCs). Each point represents the value of pooled triplicate samples per condition and data represents one of three separate experiments. (B). Percent changes in chemokine transcript levels between Bb-stimulated APCs in presence/absence of IL-10. (Data was pooled from three different experiments and expressed as percent differences between values from Bb-stimulated APCs treated with anti-IL-10 mAb compared to those treated with isotype mAb at each time point. (*from each color indicates P<0.05 for its corresponding chemokines (e.g. blue = KC) in terms of differences between isotype mAb vs anti-IL-10 mAb) C). Data from three separate experiments plotted on a single graph. Each color denotes an individual experiment, with each dot representing pooled triplicate samples.
Figure 4

A

BMM

Pre-infection 15 minutes

5 minutes 30 minutes

BMDC

Pre-infection 15 minutes

5 minutes 30 minutes

Green: GFP-Bb  Red: LAMP-1  Blue: DAPI
Figure 4. Effect of IL-10 on Bb intake by APCs: APCs cultured on glass-coverslips at 37°C were infected either with GFP-Bb at 10 MOI or without as pre-infection control. At 5, 15, and 30 minutes post-infection, APCs were fixed with 4% paraformaldehyde and permeablized with triton X-100 for the staining of LAMP-1 using fluorescent antibodies. (A), Immunofluorescent images (200x) showing both BMMs and BMDCs internalizing GFP-Bb at various times post-Bb infection. Images are representative of three separate experiments, each with at least 3 different fields of views. (B), Quantitative analysis of Bb phagocytosis by APCs. APCs were infected with GFP Bb under five conditions: GFP Bb only (control), in the presence of 4ng/ml recombinant IL-10 administered overnight prior to GFP Bb infection (rIL-10), infected with non-fluorescent Bb overnight prior to GFP Bb infection (Re-infect), similar condition as Re-infect, but either in the presence of anti-IL-10 antibody (Re-infect + anti-IL-10 mAb) or an isotype control (Re-infect + isotype mAb). Percent internalization is defined as percentage of APCs per field of view containing internalized Bb 30 minutes post-Bb infection. Data shown represents the average of ten separate fields of views, each containing 75-150 APCs, comprising at least three separate experiments * Indicates values that are statistically significant (P≤0.05) compared to control cells cultured with GFP-Bb only.
Figure 5

A

![Graph showing relative fluorescent unit (RFU) over time for BMM and BMDC treated with different conditions.]

B

![Bar chart showing slope (ΔRFU/30 min) for BMM and BMDC treated with different conditions.]
Figure 5. Effects of IL-10 on Bb-induced ROS by dihydrochlorofluorescein (DCF) assay. APCs were cultured in 96-well plates with RPMI/B for 15hrs. Afterward, the cells were washed with HBSS to remove serum before resuspending cells in HBSS containing DCF. After a 15 minute pulse with DCF, cells were then pre-treated with or without rIL-10 (2ng/10^5 cells) or the NADPH oxidase inhibitor DPI for an additional 15 minutes prior to Bb stimulation. A 0.01% H₂O₂ was used as positive control for the DCF assay. ROS production was assessed by detecting the relative fluorescence intensity (RFU) value for up to 40 minutes post-stimulation. Data obtained are expressed as (A): Raw RFU values measured per minute over a 40 minute interval (Left: BMM, Right: BMDCs), (B): Slope of RFU for each condition as defined by changes in RFU over time for the first 30 minutes. Data are representative of three separate experiments. Statistically significant (P<0.05) values are indicated compared to unstimulated control (*) or stimulated without rIL-10 (**).
Figure 6

![Bar charts showing nitrite levels in BMM and BMDC](image)

- **BMM**
  - Untx: White bar
  - Bb: Black bar
  - * indicates statistical significance

- **BMDC**
  - Untx: White bars
  - Bb: Black bars

Legend:
- White bar: isotype mAb
- Black bar: anti-IL-10 mAb
Figure 6. Effects of IL-10 on Bb-induced nitric oxide production by APCs: Cultured APCs in RPMI.B were stimulated with live Bb in either the presence of anti-IL-10 or isotype control antibodies for 24 hours. Afterwards, the supernatants were harvested and nitrite levels were assessed using the Griess assay. Statistically significant (P<0.05) values are indicated between Bb stimulated versus unstimulated APCs (*)
Figure 7

A

BMM

Green = wt unstim  Red = wt + Bb  Shaded purple = Isotype mAb
Blue = IL-10 B  Orange = IL-10 B + Bb

CD86

CD40

CD80

MHCII

Plot of Mean Fluorescent Intensity (MFI)
B

BMDC

Green = wt unstim
Blue = IL-10⁻/⁻ unstim
Red = wt + Bb
Orange = IL-10⁻/⁻ + Bb

Shaded purple = Isotype mAb

---

Plot of Mean Fluorescent Intensity (MFI)
Figure 7. Effect of IL-10 on Bb-elicited upregulation of surface co-stimulation molecule expression on APCs. APCs expanded from wt and IL-10^{-/-} mice were cultured with or without live Bb for 24 h before staining with fluorescence antibodies specific for the indicated surface molecules and analyzed by FACS. (A: BMMs, B: BMDCs) Top: Histograms of the representative FACS profiles for each surface marker. Each color line represents a different condition (e.g. Bb stimulated or control). Data shown are representative of at least three experiments. Bottom: Mean fluorescent intensity (MFI) for each individual surface molecule. Each bar represents duplicate samples and data is representative of at least three separate experiments. Statistically significant (P<0.05) values are indicated compared to unstimulated (*) or stimulated WT APCs (**). C. FACS histogram of CD40 and CD80 profiles representative of BMMs stimulated with either Bb or a combination of gamma-interferon and LPS for 24h.
Chapter 3

*Borrelia burgdorferi* elicits dysregulated IL-10 production by macrophages via a phagocytosis-independent and TLR2-dependent pathway that is distinct from pro-inflammatory responses

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Abstract

*Borrelia burgdorferi* (Bb) is a tick-borne spirochete that causes Lyme disease. Bb efficiently evade host defenses and persist in host tissue despite eliciting strong immune responses that involve Toll-like receptor 2 (TLR2)-mediated events. Virulent Bb are known to rapidly elicit substantial IL-10 production by skin-resident antigen presenting cells (APCs) such as macrophages (MØs), and blocking IL-10 significantly enhances Bb clearance and APC immune functions. We hypothesize that the mechanisms involved in the dysregulated IL-10 response are initiated rapidly and distinct from those involved in proinflammatory responses. To begin to address this, BMMs were pretreated with cytochalasin D (CytoD) to block Bb phagocytosis and/or antibodies that specifically neutralize TLR2 function, thus allowing delineation of early responses to Bb. Our results demonstrate that MØs produce significant IL-10 and IL-6 levels in response to Bb independent of phagocytosis events, whereas most pro-inflammatory mediators required phagocytosis. IL-10 produced in response to intact Bb was only partially reduced after TLR2-blockage, while IL-10 elicited by the prototypic Bb lipoprotein, OspA, was completely abrogated by TLR2-blockage. Our findings demonstrate that the interactions required for the dysregulated IL-10 response only require surface interactions with Bb, and that these events are at least partially dependent on TLR2-mediated signaling. These findings suggest that additional surface receptors that interact with TLR2 may be involved in this IL-10 production and that signaling pathways independent of those required for proinflammatory mediators my be identified and targets for Lyme disease therapies.
Introduction

Lyme disease (LD) is an emerging infectious disease that is currently the leading tick-borne illness throughout both North America and Europe. The primary pathogenic organism for LD is the Gram-negative spirochetal bacterium *Borrelia burgdorferi* (Bb) (Burgdorfer, Barbour et al. 1982; Steere, Coburn et al. 2004). Bb is an obligate host parasite lacking many genes essential for the biosynthesis of nutrients, and thus must dwell within a living vertebrate or arthropod host. Infection is only known to be spread from host to host using Ixodes ticks as an immediate vector. Early symptoms of LD include fever and malaise and the characteristic bulls-eye rash known as erythema migrans (Dandache and Nadelman 2008). While these symptoms dissipate quickly, the bacterial pathogen may persist within various host tissues for a long period of time. The re-emergence of Bb from these tissues is responsible for secondary disease symptoms such as arthritis, endocarditis, and neuroborreliosis (Bratton, Whiteside et al. 2008). Like most Gram-negative organisms, Bb lacks a typical thick peptidoglycan/teichoic acid cell wall, Bb and other spirochetes differs from most Gram-negative bacteria in possessing an outer membrane devoid of lipopoly- or lipooligo-saccharides. However, the outer membrane of Bb is rich in tri-acylated lipoproteins (Brandt, Riley et al. 1990) that activate many different host innate immune cell types, thus producing the inflammatory pathology characteristic of LD.

The mechanisms by which Bb persists within an immune-competent host for a long period of time are largely unknown and identification of those mechanisms is of great interest for designing therapies for LD.
Several major immune effectors that are critical for control of other pathogens appear to have minimal effects on Bb clearance; these include CD4- and CD8- positive T cells (Barthold, Sidman et al. 1992; Barthold and Bockenstedt 1993), the complement system (Woodman, Cooley et al. 2007) and nitric oxide (Brown and Reiner 1999). Bb-specific antibodies are only effective during the early stages of Bb infection (Barthold and Bockenstedt 1993), and the natural development of Bb-specific antibodies appears too late to eradicate infection. Some strategies that Bb utilizes to evade immune clearance have been described. Bb produces a variety of proteins that can sequester the host regulatory molecule factor H to evade complement-mediated killing (Woodman, Cooley et al. 2007), and possesses a \(vlsE\) gene that can “swap out” different genetic cassettes that allowing immense antigen variation in this surface expressed lipoprotein. Only a few innate immune mediators are critical for Bb clearance. The best known example is Toll-like receptor 2 (TLR2), where the loss of TLR2 signaling results in a substantial decrease in Bb clearance (Wooten, Ma et al. 2002). These effects appear due to interactions with Bb lipoproteins containing the tripalmitoy-S-glyceryl cysteine (PAM\(_3\)Cys) motif, and these signaling results in a variety of pro-inflammatory mediators from several cell types, including macrophages, that promote host innate immune responses. (Hirschfeld, Kirschning et al. 1999; Alexopoulou, Thomas et al. 2002; Wooten, Ma et al. 2002).

One mechanism that Bb utilizes to evade immune-clearance is by dysregulating the production of IL-10 by infected hosts. IL-10 is a potent anti-inflammatory cytokine that is normally produced later during an infection/inflammatory response to down-regulate inflammation, thus preventing damage to host tissues (Moore, de Waal Malefyt et al. 2001; Couper, Blount et al. 2008; Mosser and Zhang 2008). Deficiencies in IL-10
are associated with many inflammatory disorders, such as various forms of inflammatory bowel disease (Leach, Davidson et al. 1999; Uhlig, Coombes et al. 2006), several forms of arthritis, and other autoimmune conditions (Isomaki, Luukkanen et al. 1996; Chung, Liu et al. 2007). Due to the ability of IL-10 to suppress many immune responses, certain pathogens have devised mechanisms to upregulate this cytokine to better escape immune clearance. Some examples include bacteria of the *Mycobacteria* (Murray and Young 1999) and *Bordetella spps.* (Ross, Lavelle et al. 2004), protozoal pathogens such as *Leishmania spps* (Belkaid, Hoffmann et al. 2001), various viral pathogens (Brooks, Trifilo et al. 2006) and certain nematodes (Figueiredo, Hofer et al. 2009). Previous studies have demonstrated that host IL-10 production can directly suppress Bb clearance from host tissues, such that mice deficient in IL-10 production (IL-10⁻/⁻) show significantly reduced Bb numbers in target tissues compared to wild type (WT) mice (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). In addition, IL-10⁻/⁻ mice require an eight-fold higher infectious dose (ID₅₀) of Bb than do wild type (wt) mice (Lazarus, Meadows et al. 2006). *In-vitro* analyses indicate that both viable Bb and/or its lipoproteins (*e.g.* OspA) can rapidly elicit IL-10 production from cultured bone marrow-derived macrophages (BMM) (Wooten, Ma et al. 2002; Lazarus, Kay et al. 2008). Rapid IL-10 up-regulation was also observed *in-vivo,* near the site of Bb injection into murine skin tissues (Lazarus, Kay et al. 2008). Together, these findings indicate that IL-10 is elicited rapidly after Bb infection, and this is associated with suppression of various host innate responses (Chapter 2) that could explain Bb evasion of immune clearance.

The mechanisms that allow Bb dysregulation of host IL-10 production remain to be understood, though an important clue is that Bb lipoproteins can elicit IL-10
production by BMMs \textit{in vitro} through a TLR2-dependent mechanism (Wooten, Ma et al. 2002). The abundance of immunogenic lipoproteins on the surface of Bb is consistent with involvement in the Bb-elicited IL-10 response by BMMs. Among TLR members, TLR2 appears to be most associated with IL-10 production (Dillon, Agrawal et al. 2004; Moreira, El Kasmi et al. 2008), even though its best-described function is to elicit pro-inflammatory cytokines (Underhill and Ozinsky 2002); suggesting a dual role of TLR2 in the production of IL-10 and pro-inflammatory cytokines in response to Bb infection. However, differences in binding affinity of TLR2 for its various ligands can contribute to differential cytokine expression (Hu, Chakravarty et al. 2008). In macrophages, the process of phagocytosis can result in a recruitment of surface TLR2s to phagolysosomes, where it can cooperate with other TLR members to enhance pathogen recognition (Ozinsky, Underhill et al. 2000; Underhill and Ozinsky 2002). In fact, intracellular processing and lysosomal degradation by macrophages, of TLR2-eliciting bacteria such as the \textit{Staphylococci} spp is required for the subsequent production of cytokines (Ip, Sokolovska et al. 2010). Previous studies suggest that production of Bb-induced pro-inflammatory cytokines, by macrophages, requires phagocytosis (Moore, Cruz et al. 2007; Shin, Isberg et al. 2008; Cervantes, Dunham-Ems et al. 2011). However, whether IL-10 production in response to Bb also requires phagocytosis remains unknown.

In these studies, we intend to determine the importance of phagocytosis and TLR2 signaling in the production of IL-10 by BMMs in response to Bb, as well as comparing the elicitation of IL-10 with that of the pro-inflammatory mediators. We hypothesize that Bb induces IL-10 production by BMMs through the immediate recognition of Bb or its products on the surface of BMMs and that this interaction is TLR2-dependent. This study
will serve as the first step in understanding the signaling mechanisms involved in the
dysregulated elicitation of IL-10 production from host cells by Bb.

**Material and Methods**

*B. burgdorferi* growth and viability: A clonal N40 isolate of Bb *sensu-stricto* was
generously provided by Steve Barthold (U.C. Davis) (Barthold, Beck et al. 1990). These
bacteria were maintained *in vitro* using Barbour Stoenner and Kelly media II (BSK-II). A
clonal B31 isolate of Bb expressing green fluorescent protein (GFP) on an Erp promoter
was kindly provided by Brian Stevenson (U. Kentucky) (Miller, von Lackum et al. 2006)
and were maintained *in vitro* using BSK-II + 2% kanamycin (BSK²). All *in vitro*
experiments were performed using the bacteria that are between passages 5-7 *ex vivo*.

**Reagents and inhibitors:** Cytochalasin D (CytoD), an inhibitor for F-actin
polymerization, was purchased from Fisher Scientific. Anti-TLR-2 antibody clone T2.5,
which specifically blocks function of murine TLR-2 (Meng, Rutz et al. 2004) was
obtained from eBiosciences. Recombinant Bb lipoprotein OspA was obtained from
Merial. An ultrapure *Escherichia coli* LPS was purchased from List Biologicals. The
optimal doses used for these reagents demonstrated no cytotoxic effects (≤1%) on cell
populations as indicated by trypan blue assays. The *Escherichia coli* K12 strain was a
kind gift of Dr. Robert Blumenthal (University of Toledo).
Animal usage: C57BL/6 wild-type mice were obtained from Charles River Laboratories (NCI-Frederick). TLR2−/− mice on a C57BL/6 background were a kind gift of Dr. Marcia McInerney (University of Toledo). All animals were housed in the Department of Lab Animal Medicine at the University of Toledo Health Science Campus according to National Institute of Health guidelines for the care and use of laboratory animals. All usages protocols were reviewed and approved by the Institutional Animal Care and Usage Committee.

Expansion of murine bone-marrow macrophages (BMMs): Bone-marrow derived macrophages were produced using our previously described protocols (Lazarus, Meadows et al. 2006). Briefly, dissociated marrow tissues from the limb bones of healthy C57BL/6 WT or TLR2−/− mice were isolated and cultured for 6 days in RPMI media containing 30% L929 supernatants. At the end of the culture period, the adherent cells collected were verified as naïve BMMs (i.e. high expression of CD11b and F4/80, low expression of MHC class II and CD11c). These naïve BMMs were seeded onto tissue culture plates in culture media composed of RPMI+10%FBS supplemented with 20% BSK-II (RPMI.B) for at least 8 hours prior to beginning in-vitro assays.

In vitro co-culture of Bb with BMMs: BMMs were seeded in 24-well tissue-culture treated plates at 2-3x10^5 cells/ml. The optimal concentrations used to stimulate BMMs were: viable Bb (passage 5-7) at a multiplicity of infection (MOI) of 10, E. coli strain K12 heat-inactivated for 40 minutes at 65°C at MOI of 10 to 500, rOspA at 200ng/ml, or LPS at 3μg/ml. Where applicable, BMMs were pre-treated with specific inhibitors or
blocking antibodies for 50 minutes prior to stimulation. Immediately after stimulation, the culture plates were centrifuged at 300g for five minutes to facilitate contact between Bb and APC. Co-cultures were then incubated in a CO₂ incubator at 37°C for the indicated times.

**Detection of secreted cytokines:** Supernatants from cultured BMMs were collected at various times post-infection and cytokine content was determined by sandwich ELISA using our previously described protocols (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). All antibody pairs (including capture mAbs and biotinylated detection mAbs) and recombinant cytokine standards were purchased from BD Biosciences, and avidin-horseradish peroxidase was purchased from Vector Laboratories.

**Detection of RNA transcripts:** RNAs from BMMs co-cultured with Bb were harvested from triplicate wells of 24-well plates each containing 3x10⁵ – 4x10⁵ cells using RNeasy kit (Qiagen) and pooled to ensure sufficient sample size. Total RNA was then reverse transcribed into cDNA using ImProm II reverse transcriptase (Promega). These cDNA were quantified using a Light Cycler (Roche) rapid fluorescence temperature cycler to perform real-time quantitative PCR as previously described (Lazarus, Meadows et al. 2006). The primers used for PCR analyses were designed using software designed by Integrated DNA Technologies (IDT) and purchased through IDT.

**Determination of reactive nitrogen intermediates (RNI):** Supernatants from BMMs co-cultures were collected after overnight stimulation and nitrite (NO₂) production was
determined using the Griess method from past literature (Ding, Nathan et al. 1988), and as described in Chapter 2.

**Measurement of reactive oxygen species (ROS) generation in BMMs:** BMM-generated ROS was assayed as described previously (Chapter 2). Briefly, dichlorodihydrofluorescein-diacetate (DCFH-DA, Molecular Probes) was loaded into BMMs for 15 minutes, and BMMs were then pre-treated with or without CytoD for 30 minutes prior to Bb stimulation. After stimulation, the co-cultures were briefly centrifuged to ensure contact and the intracellular ROS was measured as relative fluorescence units (RFU) at 480nm excitation/520nm emission using a Fluostar® multi-functional plate reader (BMG).

**Flow cytometric analysis of BMM surface markers:** BMMs were seeded in 6-well plates at a density of $10^6$ cells/well. After overnight co-culture, BMMs were washed with ice-cold PBS to promote removal from the culture plates, collected into 12x75 tubes and briefly treated with 2% paraformaldehyde to fix the cells, and then pre-treated with Fc receptor-blocking antibodies. Primary antibodies to the indicated surface markers were purchased from BD Pharmingen as directly conjugated to either phycoerythin (PE) or FITC.

**Immunofluorescence microscopy (IM) imaging and phagocytosis assay:** BMMs were seeded in 12-well plates with glass cover slips within each well at a concentration of 2-3x$10^5$ cells per well in the presence or absence of cytochalasin D (Fisher Scientific).
GFP-expressing B31 Bb was added to each well (MOI = 10) and centrifuged at 300g for 3 min to facilitate Bb-BMM contact. After 30 minutes, the BMMs were washed and fixed in 4% paraformaldehyde (Fisher Scientific) overnight. To stain for compartments expressing lysosomal associated membrane protein-1 (LAMP-1), ID4B antibody (Developmental Hybridoma, U of Iowa) was used as primary antibody along with TRITC-labeled secondary goat anti-rat antibody (Southern Biotech), and DAPI was used to stain nuclei. Stained BMM-containing cover slips were mounted onto glass slides using Fluoromount® (Southern Biotech) and subsequently examined either using an epi-fluorescent microscope (FSX-100, Olympus) or a confocal fluorescence microscope (IX-81, Olympus).

**Statistical analysis:** The statistical significance of the quantitative differences between the different sample groups was determined by application of Student’s t test (two-tailed, two sample equal variance). P values of ≤0.05 were considered to be statistically significant.
Results

Production of Bb-elicited IL-10 by BMMs is phagocytosis independent, whereas most pro-inflammatory cytokines are phagocytosis dependent. To test whether IL-10 production by BMM in response to viable Bb is phagocytosis dependent, BMMs were pre-treated with cytochalasin D (CytoD), an inhibitor of F-actin polymerization, for 45 minutes prior to co-culture with Bb. Fluorescence microscopy (Figure 1A) verified that the CytoD treatment prevented Bb uptake by BMMs (Bb are external and linear), whereas untreated BMMs rapidly internalized and trafficked the tightly-coiled Bb, as previously described (Chapter 2). Internalization was further confirmed by DIC image-overlay onto fluorescence images, as well as confocal analyses (data not shown). We next measured the level of cytokines produced by BMMs in response to Bb stimulation with or without CytoD pre-treatment. In the absence of CytoD, overnight co-culture with Bb elicited substantial production of all cytokines assessed, including TNFα, IL-12, IL-6, and IL-10 (Figure 1B). However, BMM co-cultures in the presence of cytoD were unable to produce TNFα and IL-12, but were able to secrete IL-10 and IL-6 at levels similar to those not treated with CytoD. These findings indicate that phagocytosis of Bb is not necessary to elicit IL-10 production by macrophages, as opposed to elicitation of prototypical proinflammatory cytokines.

We next wanted to expand these analyses to assess the importance of phagocytosis on Bb-elicitation of chemokines involved in phagocyte recruitment during the early stages of Lyme disease. Similar to the cytokine analyses, figure 2A indicates that all of the chemokines assessed were substantially upregulated by macrophages in
response to Bb, as assessed by RT-PCR analyses; these include KC, MIP-1\(\alpha\), and MIP-2 (Fig 2). However, CytoD-treated macrophages were unable to upregulate the transcript levels of many of these chemokines in response to Bb, as was seen with transcripts of TNF\(\alpha\) and IL-12, as well as iNOS upregulation. In contrast, the CytoD treated macrophages were able to upregulate the transcript levels of IL-10 and IL-6 to levels similar to those of vehicle-treated macrophages. In summary, phagocytosis of Bb was not necessary to elicit upregulation of IL-10 or IL-6 transcripts levels by macrophages, whereas all other chemokines and proinflammatory mediators assessed required phagocytic trafficking.

**Bb phagocytosis is required for ROS and NO production, as well as upregulation of co-stimulatory molecules:** We next wanted to assess the importance of Bb phagocytosis on the production of mediators involved in intracellular killing, and in upregulation of co-stimulatory surface molecules that are important for macrophage interactions with T cells. Co-culture of Bb with macrophages elicited a strong NO response, as assessed by Griess analysis (Figure 3A), but this increase was completely suppressed in the presence of CytoD. These data are consistent with the trend observed for iNOS transcript upregulation (Figure 2), and further suggest that phagocytosis is required for NO production in response to Bb. Similarly, ROS were significantly upregulated by macrophages co-cultured with Bb, but substantially lower ROS production was observed when the co-cultures were pre-treated with CytoD (Figure 3B). Finally, CD86 was significantly upregulated by macrophage in response to Bb, similar to our previous studies in Chapter 2. However, pretreatment with cytoD completely prevented
upregulation of CD86 over background levels (Figure 3C). Overall, these findings indicate that, with the exception of IL-10 and IL-6 production, all of the assessed immune activation events associated with Bb activation of BMM require bacterial uptake/processing.

**IL-10 production in response to Bb or *E. coli* occurs independently of phagocytosis.** To determine whether the phagocytosis-independent production of IL-10 was unique to Bb activation, we compared the effects of cytoD-inhibition on cytokine production to that of heat-killed *E. coli*. Bb and *E. coli* both strongly stimulated production of most cytokines and chemokines (Figures 4A and B, respectively) by BMMs after overnight exposure to Bb. However, Bb yielded significantly higher levels of most assessed mediators, other than TNFα, compared to *E. coli* at a similar MOI. Pretreatment of BMMs with cytoD resulted in significantly lower elicitation of TNFα in response to *E. coli*. Notably, the BMM production of IL-10, KC, and MIP-2 in response to *E. coli* appeared insensitive to cytoD. This result differed from BMM responses to Bb, and suggests that *E. coli* can elicit these cytokines in the absence of phagocytosis. However, cytoD-treated BMMs showed no inhibition of IL-10 and IL-6 production in response to *E. coli*, similar to what was seen in parallel cytoD-treated Bb-stimulated BMMs. Taken together, these data indicate that *E. coli* differ from Bb by their ability to elicit many proinflammatory cytokines in the absence of phagocytosis, although they are similar in their abilities to elicit IL-10 and IL-6 in a phagocytosis-independent manner.
Production of Bb-elicited IL-10 by BMMs is at least partially dependent on TLR2
signaling. TLR2 plays an important part in mediating the inflammatory responses
involved in the development of Lyme disease (Wooten, Ma et al. 2002). To better
delineate the role of TLR2 in Bb-elicitation of IL-10, BMMs were pretreated with either
a TLR2-blocking antibody or an isotype control, and their effects on production of key
inflammatory mediators were assessed. While pretreatment of BMMs with the isotype
control antibody has no effect on Bb elicitation of any assessed mediators, treatment with
the TLR2-blocking antibody significantly suppressed the production of almost all
cytokines (Figure 5A) and chemokines (Figure 5C) assessed, as well as the production of
NO (Figure 5B). Surprisingly, the production of IL-12 appeared to be uniquely
unaffected by blocking TLR2, indicating that the strong upregulation of this key cytokine
by Bb occurs independent of TLR2 signaling. Notably, the depressed cytokine levels
observed after blocking TLR2 were still substantially higher than those exhibited by
unstimulated BMMs (Figure 5A-C). Parallel experiments, comparing Bb activation of
BMMS expanded from WT and TLR2−/− mice yielded results identical to those obtained
using the blocking antibodies (Figure 6 and data not shown). These findings suggest that
TLR2 signaling is necessary for optimal production of most Bb-elicited inflammatory
mediators; however additional receptors/signaling pathways appeared to contribute to
these activities.

TLR2-mediated production of Bb-elicited IL-10 occurs through TLR2 expressed on
the extracellular surface. TLR2-mediated signaling responses by macrophages have
been shown to occur both on the cell surface and within endosomes or phagolysosomes
Bb lipoprotein-elicitation of IL-10 by BMMs is completely dependent on TLR2-mediated signaling. Thus far, the data suggests that viable Bb elicit IL-10 production largely via cell surface TLR2 signaling, though TLR2 blockage did not completely abrogate IL-10 to baseline levels. Outer membrane lipoproteins are considered to be the main agonists possessed by Bb and these tri-acylated lipoproteins are reported to signal through a TLR2-TLR1 complex. To determine whether Bb lipoproteins are the main agonists responsible for IL-10 production, cytokine expression was compared between BMMs stimulated with viable Bb or a recombinant prototypic Bb lipoprotein, OspA.
Addition of OspA elicited both IL-10 and IL-6 from WT BMMs, but this production was reduced to background levels in the absence of TLR2 (Figure 7A), thus differing from the significant but incomplete suppression observed in TLR2−/− macrophage responses to intact Bb. OspA was similarly able to elicit IL-10 and IL-6 from BMMs in the presence and absence of CytoD, indicating that the TLR2 signaling events to the purified lipoprotein can occur on the macrophage surface, independent of phagocytosis. Interestingly, the OspA-elicited production of both IL-10 and IL-6 by BMMs pre-treated with CytoD was significantly greater than those produced by control BMMs; indicating that, similar to what was observed with *E. coli*, OspA induced greater levels of IL-10 and IL-6 by BMMs that are not capable of phagocytosis versus normal BMMs. Unlike intact Bb, OspA was unable to elicit IL-12 production from BMMs, suggesting that intact Bb elicit IL-12 production via one or more agonists other than lipoproteins; this finding is consistent with our finding that IL-12 production could occur in the absence of TLR2 (Figure 5). OspA was also capable of eliciting the production of pro-inflammatory chemokines such as KC and MIP2 from BMMs and this elicitation is also completely-dependent on TLR2 (data not shown). Interestingly, the elicitation of KC and MIP2 by OspA is independent of phagocytosis, a property that differs from that of intact Bb (Figure 7B). Together, these data suggest that lipoprotein-stimulated signaling via TLR2 is largely responsible for Bb-elicitation of IL-10, but other agonists and/or receptor ligands exist that complement these stimulatory effects.
Discussion

The abnormally high levels of IL-10 produced by murine hosts upon infection by Bb have been shown to suppress immune clearance by the host (Lazarus, Meadows et al. 2006; Lazarus, Kay et al. 2008). Due to the rapid and pronounced IL-10 elicitation, we hypothesize that the initial receptor-mediated signaling events driving this response may occur after only minimal interaction with receptors on the macrophage surface, and may represent a different signaling mechanism from those responsible for producing proinflammatory mediators. Our findings in these studies suggest that interactions of Bb with the outer surface of BMMs are sufficient for the elicitation of IL-10. On the other hand, the production of pro-inflammatory cytokines, such as IL-12 and TNFα, in response to Bb was all phagocytosis-dependent; which is consistent with other published studies (Moore, Cruz et al. 2007; Shin, Isberg et al. 2008). In other words, elicitation of most pro-inflammatory cytokines requires the internalization and/or further processing of Bb by the BMMs. Furthermore, the elicitation of other immune mediators such as nitric oxide, chemokines important for neutrophil-recruitment, and the expression of co-stimulation molecules such as CD86 by Bb, all required internalization of Bb by BMMs. Since phagocytosis is a time and energy-consuming process, IL-10 production by the hosts in response to Bb can occur at a more rapid rate than most pro-inflammatory mediators, and thus more rapidly suppress the production of these mediators, dysregulating the inflammatory response. Interestingly, our data also indicates that the production of IL-6 by BMMs in response to Bb is also independent of phagocytosis. IL-6 is a pleiotropic cytokine that exhibits functions that can be considered both
proinflammatory and anti-inflammatory, and its most abundant production is by antigen presenting cells such as BMMs (Scheller and Rose-John 2006). However, the actual role of IL-6 in Bb infection is undetermined, and is beyond the scope of this study.

Bb survives extracellularly within susceptible immunocompetent hosts from months to years post-infection, whereas non-pathogenic bacterial strains, such as *E. coli* K12, elicit immune responses that result in much more efficient host clearance (Svanborg, Bergsten et al. 2006). Both Bb and *E. coli* can elicit IL-10 production from BMMs independent of phagocytosis. However, for the production of pro-inflammatory cytokines by BMMs, Bb must be internalized and/or processed, whereas *E. coli* can still elicit substantial levels of proinflammatory mediators independent of phagocytosis.

Others have also observed that elicitation of cytokines in general from BMMs by *E. coli* requires only interaction on the cell surface (Ip, Sokolovska et al. 2010). This suggests that there are differences in surface molecules between the two bacteria, where for Bb; the extracellular components only elicit a minor subset of soluble mediators, which include IL-10. Thus, it is possible that this delay in producing the signals required for proinflammatory mediators, in addition to the rapid upregulation of IL-10, may further aid Bb evasion of early immune clearance. The ability of Bb to elicit IL-10 production from BMMs prior to being phagocytosed suggests that BMM surface pattern-recognition receptors are involved in transducing the signals necessary for Bb-elicitation of IL-10 production. TLR2 is the best described receptor involved in Bb-induced immune responses from macrophages (Shin, Isberg et al. 2008; Sahay, Patsey et al. 2009; Salazar, Duhnam-Ems et al. 2009). In this study, we demonstrated that the elicitation of IL-10 by viable Bb involves TLR2. Besides IL-10, the production of most cytokines, chemokines,
and other inflammatory mediators we assessed were also TLR2-dependent. These findings were consistent with others indicating that TLR2 is important for Bb sensing by macrophages.

Interestingly, the production of IL-12 in response to Bb was independent of TLR2. Generally, strong IL-12 responses are more often associated with macrophages infected with intracellular pathogens, such as *Toxiplasma gondii* and *Leishmania* spp, which also elicit IL-12 independently of TLR2 (Yarovinsky 2008; Vargas-Inchaustegui, Tai et al. 2009). This observation that Bb exerts a similar pattern of IL-12 elicitation is interesting since Bb is not known to thrive intracellularly for any extended time. It is also notable that most of the stimulatory properties for Bb are associated with their outer membrane lipoproteins, since they lack LPS, lipoteichoic acids, external flagella, or other notable bacterial agonists that would elicit stimulatory responses via pattern recognition receptors. Thus, the agonist responsible for eliciting the IL-12 response to Bb is currently unknown.

Although blocking/depleting TLR2 signaling resulted in suppressed IL-10 responses against intact Bb, as well as decreasing many other cytokines/chemokines, this suppression is usually incomplete (*i.e.* the levels were still substantially above background). This finding, along with the observation that Bb-elicitation of IL-12 was completely independent of TLR2-signaling, indicates that other receptors besides TLR2 are important for Bb-sensing. Other members of the TLR family, such as TLR3, TLR5, and TLR9, participate in the sensing of various pathogens, although these receptors have not been reported to play a substantial role in Bb-induction of cytokine production (Shin, Isberg et al. 2008). Recently, it has been reported that intracellular TLRs, such as TLR8,
play a role in the production of IL-10 by BMMs in response to Bb (Cervantes, Dunham-
Ems et al. 2011). In addition to TLRs, other pattern-recognition receptors, such as the
nucleotide-oligolization domain-2 (NOD2) family receptor, have been reported to
associate with TLR2 to promote IL-10 production (Moreira, El Kasmi et al. 2008). In
fact, there is evidence that NOD2 is involved in immune-sensing of Bb (Miller, Maylor-
Hagen et al.; Oosting, Berende et al. 2010). Although NOD2 is currently believed to
signal in response to cytoplasmic agonists, while our findings indicate that Bb can elicit
IL-10 without being internalized by macrophages, NOD2 could potentially be important
for the production of many of the proinflammatory mediators, particularly IL-10. Our
findings also indicate that TLR2 is not the only receptor on the macrophage surface that
is responsible for IL-10-elicitation in response to intact Bb. One possibly relevant
surface receptor is TLR1, since TLR1 cooperates with TLR2 in sensing tri-acylated
lipoproteins (Schenk, Belisle et al. 2009). In addition to TLR family receptors, scavenger
receptors such as CD36 to facilitate TLR2 signaling on macrophages in response to host-
derived lipoproteins (Park, Febbraio et al. 2009). Notably, macrophages have been
reported to upregulate IL-10 via CD36 ligation of host lipoproteins (Chung, Liu et al.
2007), making this receptor a possible candidate to promote IL-10 production in response
to Bb lipoproteins.

The active moiety on Bb surface lipoproteins responsible for activating via TLR2
is the PAM3Cys motif, that is present on the amino terminal of all Bb lipoproteins (Ma,
Seiler et al. 1994). Our current studies demonstrate that the prototypical Bb lipoprotein
OspA is capable of eliciting IL-10 production from BMMs, and that this IL-10
production is completely dependent on TLR2 signaling; a finding that is consistent with
previous studies (Wooten, Ma et al. 2002). Since the elicitation of IL-10 by intact Bb was only partially dependent on TLR2, our data suggest that there are other components besides lipoproteins that are instrumental in this event. As with intact Bb, the elicitation of IL-10 and IL-6 by Bb lipoproteins also occurs on the surface of BMMs prior to phagocytosis. However, the elicitation of many pro-inflammatory mediators, such as KC and MIP-2, from BMMs by Bb lipoproteins were also independent of phagocytosis. One possible hypothesis is that intact Bb needs to be degraded by BMMs intracellularly before they can signal for the production of pro-inflammatory cytokines, whereas it is unnecessary for isolated Bb lipoproteins. Preliminary data from our lab examining the role of lysosomal acidification on the production of various cytokines by BMMs in response to intact Bb versus Bb lipoproteins indicated that pretreating BMMs with chloroquine, an inhibitor of lysosomal acidification, yielded suppressed levels of cytokine production in response to intact Bb, whereas the levels of cytokine production in response to Bb-lipoproteins remain unchanged; a finding that supports this hypothesis (Chung et al. unpublished data). Interestingly, Bb lipoproteins elicited very little IL-12 production from BMMs as compared to intact Bb. Since Bb-elicited IL-12 was also shown to be dependent on phagocytosis, but independent of TLR2, our results indicate that there are additional unknown immune agonists expressed by Bb that can activate BMMs from within.

Our current model for Bb elicitation of IL-10 is based on our central findings that: the signals required for Bb-elicitation of IL-10 by BMMs occurs on the outer surface through a TLR2-dependent manner, whereas the elicitation of pro-inflammatory cytokines requires Bb to be internalized and sensed through TLR2 within phagosomal
compartments (Figure 8). During an actual Bb infection, it has been speculated that the relatively high velocity of Bb movement within host tissues allows the bacteria to evade capture by host immune cells. Intravital imaging studies from our lab indicated that the vast majority of Bb residing within skin tissues were found to be “swimming” around different APC populations without being phagocytosed, but also leaving behind small membrane blebs that likely possess a population of surface lipoproteins (Wooten, unpublished). We and others have also observed Bb in extended contact with certain APCs both in vitro and in vivo (Wooten, unpublished), where the APCs are attempting to internalize the spirochetes, while the bacteria continue to struggle to escape phagocytosis, and often are successful in escaping. Overall, these extended in vivo interactions between Bb and macrophages, together with the lesser requirements of interactions that lead to IL-10 production, may further add to the large and potent IL-10 response seen early during infection, and provide an advantage to allow early escape of Bb from the innate immune responses.
Figure 1

A

X-Y plane

Control

Cyto D

Y-Z plane

Green = GFP Bb  Red = LAMP1  Blue = DAPI

B

IL-10  IL-6

vehicle cytoD

IL-12  TNFα
Figure 1: Bb elicitation of IL-10 by BMMs is independent of Bb-phagocytosis.

BMMs were pre-treated with 2μM CytoD or 0.1% DMSO (vehicle) for 45 min prior to overnight stimulation with Bb (MOI = 10). (A), Confocal microscopy images of vehicle-pretreated (left panel) or CytoD-pretreated BMMs after 30 min of Bb infection (Top: XY plane, Bottom: Z-stack, scale bar = 10 μm). (B), Cytokine production by BMMs in response to Bb stimulation in the presence or absence of CytoD, as measured by ELISA. Results shown are representative of experiments performed at least five times. * indicates P<0.05 in differences between unstimulated versus Bb-stimulated BMMs.
Figure 2

A

![Graphs showing cytokine expression over time](image)

Fold-induction over baseline for various cytokines:
- IL-10
- IL-6
- IL-12
- TNFα
- KC
- MIP-2
- MIP-1a
- iNOS

B

![Graph showing relative expression over Bb only control](image)

Relative expression over Bb only control for different cytokines:
- IL-10
- IL-6
- IL-12
- TNF
- KC
- MIP2
- MIP1α
- iNOS

*Significance levels indicated.
Figure 2: Effect of phagocytosis on the transcription of various cytokines and chemokines by BMMs in response to Bb. Parallel groups of BMMs were co-cultured with Bb (MOI = 10) and groups were harvested at 4, 8, and 12 h post-stimulation for total RNA extraction. Transcript levels of cytokine, chemokine and iNOS were assessed by quantitative RT-PCR. (A), Data displayed as fold-increase over unstimulated control. Each data point is representative of pooled triplicate wells of co-cultured BMMs receiving the same treatments. (B), Data displayed as percent differences of Bb-stimulated BMMs pre-treated with CytoD compared to Bb-stimulated BMMs pre-treated with DMSO (vehicle control; set to 100 percent). Each bar represents an average of at least three separate experiments. (* indicates that P<0.05 in differences between DMSO-treated BMMs stimulated with Bb versus CytoD-treated BMMs stimulated with Bb at the indicated time points.) (C), Data from three separate experiments plotted on one graph. Each color denotes one experiment performed with each dot representing pooled triplicate samples.
Figure 3

A

![Graph showing NO production with vehicle and cytoD](image)

B

![Graph showing ROS production over time](image)

C

![Graph showing CD86 expression](image)
Figure 3: Effects of Bb-phagocytosis on production of different proinflammatory mediators. (A), BMMs pretreated with either vehicle or CytoD were co-cultured overnight with Bb (MOI = 10) before assessing the supernatants for NO production using the Griess method. (B), ROS generation by BMMs during the first 45 min of Bb stimulation with or without CytoD pre-treatment. C, BMMs pretreated with either vehicle or CytoD were co-cultured overnight with Bb (MOI = 10) before collecting the cells and assessing surface expression of CD86 by FACS analysis. (* indicates P<0.05 in differences between unstimulated versus Bb-stimulated BMMs ** indicates P<0.05 between BMMs treated with or without CytoD)
Figure 4

A

**IL-10**

- Untx
- Bb
- E coli

**IL-6**

- Untx
- Bb
- E coli

**IL-12**

- Untx
- Bb
- E coli

**TNFα**

- Untx
- Bb
- E coli

B

- E coli + DMSO
- E coli + cytoD

Relative expression over control

- KC
- MIP2
Figure 4: Comparison of the importance of phagocytosis in eliciting cytokine responses to Bb and E. coli. BMMs were stimulated with either live Bb (MOI = 10) or with heat-killed E. coli strain K12 (MOI = 10) overnight in the presence or absence of CytoD pre-treatment. (A), Cytokine production as determined by ELISA. (B), Chemokine production as determined by qRT-PCR, where relative expression is defined as percent differences between E coli-stimulated cells in the presence of CytoD versus E. coli-stimulated controls (set to 100 percent). Data are representative of three individual experiments, each performed as triplicate samples. (* indicates P<0.05 in differences between unstimulated versus Bb-stimulated BMMs, ** indicates P<0.05 in differences between CytoD versus no CytoD treatments, *** indicates P<0.05 between Bb and E. coli-stimulated BMMs)
Figure 5

A

**IL-10**

![Graph showing IL-10 levels with untx and Bb groups]

**IL-6**

![Graph showing IL-6 levels with untx and Bb groups]

**TNFα**

![Graph showing TNFα levels with untx and Bb groups]

**IL-12**

![Graph showing IL-12 levels with untx and Bb groups]

B

**NO (Griess)**

![Graph showing NO levels with untx and Bb groups]

C

**Chemokines (qRT PCR)**

![Bar chart showing relative expression of KC and MIP2 with Bb and Bb+anti-TLR2 mAb]

Legend:
- White bars: isotype mAb
- Black bars: anti-TLR2 mAb

Significance levels indicated by asterisks (*) and double asterisks (**)
Figure 5: Bb-elicitation of IL-10 by BMMs is partially dependent on TLR2. BMMs are pretreated with either an isotype control monoclonal antibody (isotype mAb) or the anti-mouse TLR2-blocking monoclonal antibody T2.5 (anti-TLR2 mAb) for 30 minutes prior to Bb stimulation. (A), Secreted cytokine production as measured by ELISA. (B), Nitric oxide (NO) production assessed by the Griess method. (C), Chemokine transcript production by BMMs as measured by qRT-PCR after eight hours of Bb stimulation and expressed as percent of Bb-stimulated BMMs treated with isotype mAb. Each bar represents a sample pooled from three separate wells. All data shown are representative of at least three separate experiments. * indicates P< 0.05 in comparisons between BMMs receiving isotype mAb versus anti-TLR2 mAb treatments. ** indicates P< 0.05 between untreated (untx) versus Bb-stimulated BMMs.
Figure 6: TLR2-mediated IL-10 and IL-6 production by BMM in response to Bb occurs on the cell surface of BMMs. (A), BMMs were pre-treated with or without CytoD for 45 minutes in the presence of either an isotype control or anti-TLR2 mAb for 30 minutes prior to Bb stimulation. (B), BMMs from WT mice or TLR2^-/- mice are pre-treated with or without CytoD for 45 minutes prior to Bb stimulation. * indicates P<0.05 either between isotype mAb treatment versus anti-TLR2 mAb treatment, or between WT versus TLR2^-/- BMMs. ** indicates P<0.05 between unstimulated BMMs versus Bb-stimulated BMMs.
Figure 7

A

![Graphs showing cytokine expression levels](image)

- **IL-10**
- **IL-6**
- **IL-12**

Legend:
- CNT
- Bb
- OspA

*CytoD - wt, TLR2^-/-*
Figure 7: Bb lipoprotein-mediated elicitation of both IL-10 and pro-inflammatory cytokines are independent of phagocytosis, but completely dependent on TLR2.

BMMs from wt C57BL/6 or TLR2^/-^ mice were stimulated with either viable Bb (MOI = 10) or recombinant OspA (200ng/ml) in the presence or absence of CytoD for 24 h. (A) Comparison of cytokine production by wt and TLR2^/-^ BMMs in response to the different agonists, in the presence or absence of CytoD. * indicates P<0.05 in differences between unstimulated and Bb-stimulated BMMs. ** indicates P<0.05 in differences between WT versus TLR2 BMMs. (B) Chemokine production by stimulated BMMs assessed by qRT-PCR in the presence or absence of CytoD. Data represents the relative levels of chemokine production in the presence of CytoD as percent of vehicle control. * indicates P<0.05 in differences between BMMs treated with or without CytoD.
Figure 8

A

TLR2

MyD88?

Macrophage

Bb lipoproteins

Bb

IL-10

B

TLR2

MyD88?

phagosome

Macrophage

Bb

Pro-inflammatory responses
Figure 8: Putative pathways for the initiation of IL-10 production versus pro-inflammatory responses by BMMs when stimulated with Bb. (A), Model pathway for Bb-elicited IL-10 elicitation, where extracellular interactions between Bb and its surface lipoproteins with TLR2 are sufficient to induce macrophages to produce IL-10. (B), Model pathway for generation of Bb-elicited pro-inflammatory responses by macrophages, where most of these responses require Bb to be phagocytosed. This model predicts that the interactions between Bb and intracellular receptors, such as internalized TLR2, present within phagosomes that are needed for generation of pro-inflammatory response against Bb by macrophages.
Chapter 4

Activation of PI3 kinase and MAP kinases are required for the production of IL-10, but not most pro-inflammatory cytokines, by murine macrophages in response to *Borrelia burgdorferi*

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Abstract

The causative agent for Lyme disease, the tick-borne spirochetal bacterium Borrelia burgdorferi (Bb), evades host immune killing despite mounting a specific immune response from the host. The ability of Bb to elicit dysregulated host production of the anti-inflammatory cytokine IL-10 is a major mechanism for evading host immune clearance and establishing persistence. We have previously demonstrated that certain innate immune cells, such as macrophages, produce abnormally-high levels of IL-10 upon contact with Bb or its lipoproteins, and that this IL-10 subsequently suppresses the elicitation of pro-inflammatory mediators. To further delineate signaling cascades most responsible for this Bb-induced IL-10 production, we have examined the role of PI3-kinase and MAP kinase pathways. Our studies demonstrate that Bb-elicited IL-10 production in macrophages is suppressed when the PI3 kinase/Akt pathways are inhibited. Similarly, inhibiting the activation of MAP kinases p38 and ERK1/2 also led to suppression of Bb-induced IL-10. Among Bb-elicited pro-inflammatory cytokines, only TNF-α yielded a result similar to IL-10 when the above pathways were inhibited, whereas the other cytokines assessed were unaffected. Activation of both the PI3-kinase and MAP kinase cascades by macrophages in response to Bb is TLR2 dependent, yet is phagocytosis independent. In vivo injection of a MAP kinase p38 inhibitor resulted in suppressed IL-10 transcript levels in the skin of Bb-infected mice, while levels of most proinflammatory mediators were relatively unaffected and also resulted in reduced Bb levels in most host target tissues. Overall, our findings indicate that PI3 kinase and MAP kinase signaling pathways are both involved in the dysregulated production of host IL-10 in response to Bb infection, and may provide targets for eradication therapies.
Introduction

*Borrelia burgdorferi* (Bb) is a tick-borne bacterium of the family Spirochaete, that is the causative agent of Lyme disease, the leading vector-borne disease in the United States (Steere, Coburn et al. 2004). Although Bb, like other spirochetes, has a Gram negative-like cell wall structure, it does not contain lipopolysaccharide in its outer membrane. Instead, the outer membrane is rich in lipoproteins containing the triacylated tripalmitoyl-S-glyceryl cysteine (PAM3Cys) motif (Brandt, Riley et al. 1990), which are known to activate certain immune cell populations through toll-like receptor 2 (TLR2) - mediated signaling. Bb is an obligate parasite that must reside within certain reservoir hosts and is only transmitted between animal hosts when infected ticks take blood meals. Upon infection, the host develops an initial inflammatory response inclusive of common cold-like symptoms and a characteristic erythema chronica migrans (EM) that abates within days (Dandache and Nadelman 2008). However, the bacteria often persist within various tissues and organs, leading to secondary diseases such as Lyme arthritis (Steere 1989; Wormser 2006). Therefore, despite a strong immune response, the pathogen is often not effectively killed or cleared from the host. Studies examining the role of individual immune effectors, such as CD4\(^+\) T cells, alternative complement pathways, and nitric oxide production, revealed only minimal effects on Bb clearance (Barthold, Sidman et al. 1992; Brown and Reiner 1999; Woodman, Cooley et al. 2007). Specific antibodies can effectively clear Bb, but only during very early stages of infection that precede the natural appearance of Bb-specific antibodies (Barthold and Bockenstedt 1993). Studies have identified several immune-evasive mechanisms for Bb, most of which are mediated by different surface lipoproteins (Fikrig and Narasimhan 2006).
Overall, the identification of functional immune effectors against Bb is a perplexing process within the field of immunology.

Currently, the cytokine interleukin 10 (IL-10) is the best known soluble immune effector to play a significant role in Bb clearance. IL-10 is an anti-inflammatory cytokine that normally functions to regulate excessive immune responses (Moore, de Waal Malefyt et al. 2001; Mosser and Zhang 2008). However, infected mice deficient in IL-10 harbor significantly lower Bb numbers in multiple tissues, and have an eight-fold greater ID$_{50}$ compared to wt mice (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). These findings indicate that host IL-10 production interferes with Bb clearance. In-vitro studies further demonstrate that both viable Bb and Bb lipoproteins elicit high levels of IL-10 production from resident antigen-presenting cells (APCs), such as bone-marrow derived macrophages (BMMs) (Wooten, Ma et al. 2002; Lazarus, Kay et al. 2008). This IL-10 can feed back on resident APCs to suppress the production of various immune mediators, including the production of pro-inflammatory cytokines, neutrophil-recruiting chemokines, reactive oxygen species, and co-stimulatory molecules, such as CD86 (Chapter 2). In vivo studies also revealed that Bb-infected mice produce high levels of IL-10 in infected skin tissues within 24 hours of injection (Lazarus, Kay et al. 2008), indicating that IL-10 is most likely produced by APCs or other skin-resident cells that interact with Bb. The ability of Bb to rapidly and potently dysregulate IL-10 production by skin-resident host cells is believed to represent an important virulence mechanism for the bacteria to evade host immune killing.

The mechanisms of pathogen-mediated dysregulation of IL-10 production are beginning to be understood. IL-10 induction by pathogens usually requires particular
pattern-recognition receptors, such as TLR2 (Dillon, Agrawal et al. 2004; Moreira, El Kasmi et al. 2008), which are also required for the elicitation of many pro-inflammatory responses (Underhill and Ozinsky 2002). TLR2-signaling is required for effective clearance of Bb, and is important for the production of pro-inflammatory mediators in response to Bb (Hirschfeld, Kirschning et al. 1999; Wooten, Ma et al. 2002; Wooten, Ma et al. 2002; Shin, Isberg et al. 2008). Recently, we demonstrated that TLR2 expressed on the surface of BMMs is also important for the induction of IL-10 production in response to both viable Bb and its lipoproteins (Chapter 3). However, the downstream signaling cascades involved in Bb-elicited IL-10 production remain to be determined.

Cytosolic signaling molecules such as phosphatidylinositol-3 kinase (PI3 kinase), and its immediate down-stream effector Akt or protein kinase B, are involved in IL-10 production in both myeloid and non-myeloid cells in response to various agonists (Fukao, Tanabe et al. 2002; Dahle, Overland et al. 2004; Gunzl and Schabbauer 2008; Mendez-Samperio, Trejo et al. 2008; Gunzl, Bauer et al. 2010). There is also evidence linking the mammalian target of rapamycin (mTOR), an immediate downstream effector of the PI3 kinase/Akt pathway (Sekulic, Hudson et al. 2000), to the elicitation of IL-10 production in response to various agonists (Luyendyk, Schabbauer et al. 2008; Weichhart, Costantino et al. 2008; Saemann, Haidinger et al. 2009). In addition to PI3 kinase-mediated signaling pathways, members of the mitogen-activating protein kinase (MAP kinase) family, such as p38 and extracellular receptor kinase 1/2 (ERK1/2; also known as p40/42), have been directly linked to the production of IL-10 by immune cells in response to both pathogen and non-pathogen stimuli (Ma, Lim et al. 2001; Martin, Schifferle et al. 2003; Chelvarajan, Popa et al. 2007; Chung, Liu et al. 2007; Mendez-
Samperio, Trejo et al. 2008; Slobedman, Barry et al. 2009; Yang, Zhang et al. 2010).
The role of both PI3 kinase and MAP kinase pathways in the production of pro-
inflammatory cytokines by myeloid cells is currently unclear. For example, the
production of TNF-α has been reported to require MAP kinase pathways whereas the IL-
12 production has been reported to be inhibited by PI3 Kinase and/or p38 MAPK (Kaji,
Kiyoshima-Shibata et al.; Martin, Schifferle et al. 2003; Chelvarajan, Popa et al. 2007).
These findings suggest that different pathogens, by preferentially activating certain
signaling cascades within host immune cells, could potentially direct the host toward
over-production of IL-10, thus allowing better evasion of the host immune responses.

In this study, we intend to characterize the key host intracellular signaling
mechanisms involved in production of IL-10 by BMMs in response to viable Bb. We
hypothesize that this induction involves both PI3 kinase and MAP kinase members
subsequent to TLR2 signaling. We also believe that the particular signaling pathway(s)
utilized for Bb-elicited IL-10 by BMMs is/are distinct from those required for production
of most pro-inflammatory cytokines. Delineating these pathways may facilitate the
designing of therapeutics against Bb infection and the development of LD.

Material and Methods

*B. burgdorferi* growth and viability: A clonal N40 isolate of Bb *sensu-stricto* was
generously provided by Steve Barthold (U.C. Davis) (Barthold, Beck et al. 1990). These
bacteria were maintained in vitro using Barbour Stoenner and Kelly media II (BSK-II). A
clonal B31 isolate of Bb expressing green fluorescent protein (GFP) on an Erp promoter
was kindly provided by Brian Stevenson (U. Kentucky) (Miller, von Lackum et al. 2006) and were maintained in vitro using BSK-II + 2% kanamycin (BSK). All in vitro experiments were performed using the bacteria that are between passages 5-7 ex vivo.

**Reagents and inhibitors:** Specific inhibitors for the PI3 kinase (LY294002 and Wortmannin), MAP kinase p38 pathway (SB203580 and SB202190), and MAP kinase ERK1/2 pathway (U0126) were purchased from both Cell Signaling and EMD Biosciences. For experiments involving inhibition of phagocytosis, the F-actin polymerization inhibitor cytochalasin D was purchased from Fisher Scientific. Recombinant Bb lipoprotein L-OspAa was obtained from Merial. LPS derived from *Salmonella typhimurium* was purchased from List Biologicals.

**Animal usage:** C57BL/6 wild-type mice were obtained from Charles River Laboratories (NCI-Frederick). TLR2−/− mice on a C57BL/6 background were a kind gift of Dr. Marcia McInerney (University of Toledo). All animals were housed in the Department of Lab Animal Medicine at the University of Toledo Health Science Campus according to National Institute of Health guidelines for the care and use of laboratory animals. All usages protocols were reviewed and approved by the Institutional Animal Care and Usage Committee.

**Expansion of murine bone-marrow macrophages (BMMs):** Bone-marrow derived macrophages were produced using our previously described protocols (Lazarus, Meadows et al. 2006). Briefly, dissociated marrow tissues from the limb bones of
healthy C57BL/6 WT or TLR2−/− mice were isolated and cultured for 6 days in RPMI media containing 30% L929 supernatants. At the end of the culture period, the adherent cells collected were verified as naïve BMMs (i.e. high expression of CD11b and F4/80, low expression of MHC class II and CD11c). These naïve BMMs were seeded onto tissue culture plates in culture media composed of RPMI+10%FBS supplemented with 20% BSK-II (RPMI.B) for at least 8 hours prior to beginning in-vitro assays.

**In vitro co-culture of Bb with BMMs:** BMMs were seeded in 24-well tissue-culture treated plates at 2-3 x 10^5 cells/ml. The optimal concentrations used to stimulate BMMs were: viable Bb (passage 5-7) at a multiplicity of infection (MOI) of 10 , rOspA at 200ng/ml, or LPS at 3μg/ml. Where applicable, BMMs were pre-treated with specific inhibitors or blocking antibodies for 50 minutes prior to stimulation. Immediately after stimulation, the culture plates were centrifuged at 300g for five minutes to facilitate contact between Bb and APC. For co-culture experiments intended to detect phosphorylation events, BMMS were seeded into 12-well plates at 7 x 10^5 cells/ml and allowed to rest for at least 10 h before stimulation. All co-cultures were incubated in a CO₂ incubator at 37°C for the indicated times.

**Detection of secreted cytokines:** Supernatants from cultured BMMs were collected at various times post-infection and cytokine content was determined by sandwich ELISA using our previously described protocols (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). All antibody pairs (including capture mAbs and biotinylated detection mAbs) and recombinant cytokine standards were purchased from either BD Biosciences
or eBiosciences, and avidin-horseradish peroxidase was purchased from Vector Laboratories.

**Detection of RNA transcripts:** RNAs from BMM co-cultures were harvested from triplicate wells of 24-well plates each containing $3 \times 10^5$ – $4 \times 10^5$ cells using RNeasy kit (Qiagen) and pooled to ensure sufficient sample size. Total RNA was then reverse transcribed into cDNA using ImProm II reverse transcriptase (Promega). These cDNA were quantified using a Light Cycler (Roche) rapid fluorescence temperature cycler to perform real-time quantitative PCR as previously described (Lazarus, Meadows et al. 2006). The primers used for PCR analyses were designed using software designed by Integrated DNA Technologies (IDT) and purchased through IDT. RNA recovered from infected mouse tissues (see below) were reverse transcribed and quantified as for the BMM-derived transcripts.

**Western Blots:** To detect the phosphorylated and total forms of PI3 kinase, MAP Kinase p38, and ERK Kinase, BMMs were seeded as described above. Cells were pre-treated for 1 h either with selected inhibitors or a vehicle controls prior to stimulation with Bb, OspA, or LPS. BMMs were harvested at the indicated times post-stimulation using a passive lysis buffer (Promega) containing Halt® Protease and the Phosphatase Inhibitor Cocktail (Thermo Scientific). Proteins were resolved on NuPAGE® 4-12% Bis-Tris gels (Invitrogen) using NuPAGE® MOPS SDS running buffer (Invitrogen). Gel proteins were transferred to 0.2μm PVDF membrane (Millipore) using NuPAGE® transfer buffer (Invitrogen) at 30V for 1.5 hour. Membranes were incubated in blocking
buffer containing 0.1% Tween-20 and 5% w/v nonfat dry milk for 1 h, then incubated with the indicated primary antibodies overnight before washing and adding the anti-rabbit IgG-HRP linked secondary Ab for 2 h. All primary and secondary antibodies were purchased from Cell Signaling. For protein detection, either ECL Western Reagents (Amersham Biosciences) or SuperSignal® West Pico Chemiluminescent (Pierce) were used to visualize protein bands on the membrane.

**In vivo infection study:** C57BL/6 mice (6-8 weeks-old) were inoculated intradermally with 2 x 10^4 N40 Bb 1 h after receiving subcutaneous injection of either water-soluble SB203580 (EMD Bioscience) at 5mg/kg animal weight or PBS as the vehicle control; this injection protocol for SB203580 was based on published (Badger, Bradbeer et al. 1996; Qian, Deng et al. 2009), as well as our pilot studies. At 30 hours post-infection, the mice were euthanized and the skin at the Bb inoculation site was removed and homogenized using the Tissuelyser® system (Qiagen). Tissue lysates were placed in Allprep® columns (Qiagen) for the extraction and purification of DNA, RNA, and proteins per the manufacturer’s specification with slight modifications to optimize recovery. These RNA transcripts were quantified as indicated above.

To determine the long-term effects of administration of p38 MAP kinase inhibitor on the clearance of Bb, C57BL/6 mice of both wt and IL-10^−/− background (6-8 weeks-old) were inoculated intradermally with 2 x 10^4 N40 Bb as described above, but maintained for three weeks. During the infection period, the infected mice from each strain were further divided into two groups of 7-8 mice, where one group received water-soluble p38 MAP kinase inhibitor SB203580 injected at 5mg/kg animal weight
subcutaneously on the day of Bb inoculation, as well as on alternate days afterward (subcutaneously for the first 10 days, intra-peritoneally for the next 10 days); while the other group received PBS as an injection control. Mice were euthanized after the three weeks period and the whole heart, the back skin near the site of injection, the left ankle, and the whole left ear were harvested from each animal. DNA was extracted from these tissues for quantitative PCR analysis to determine the Bb numbers using our previously described methods (Lazarus, Meadows et al. 2006). Briefly, mice tissues were digested using a proteinase K/collagenase mixture. Total DNA from the digested tissues were extracted using phenol-chloroform and precipitated using ethanol. Final purified DNA from each mice tissue was resuspended in 10mM Tris buffer containing 1mM EDTA. The initial DNA concentration was determined by absorbance at 260 nm wavelength and subsequently adjusted to 50µg/ml per sample. From these purified DNA samples, quantitative-PCR was performed to determine copy numbers of nidogen, a single copy mouse gene, and recA, a single copy Bb gene by extrapolation to standard curves as described previously (Lazarus, Meadows et al. 2006). Final reported data represents number of recA (i.e. Bb genomes) copies per 1000 nidogen (i.e. mouse genome) copies.

Statistical analysis: The statistical significance of the quantitative differences between the different sample groups was determined by application of Student’s t test (two-tailed, two sample equal variance). For assessing the statistical significance of the quantitative differences between the samples from the different mice groups of the in-vivo studies, a one-way ANOVA with post-test was performed. P values of ≤0.05 were considered to be statistically significant.
Results

**Bb elicitation of IL-10 by BMMs requires PI3 kinase activation.** To determine whether signaling events involving the PI3 kinase pathway are involved in the production of Bb-elicited IL-10, BMMs were co-cultured with Bb in the presence or absence of the PI3 kinase inhibitor LY294002 (LY). We then measured the effects on phosphorylation of the serine-threonine protein kinase Akt, which is dependent on PI3 kinase activity (Marone, Cmiljanovic et al. 2008). Immunoblots and densitometry analysis demonstrated that unstimulated BMMs contained low levels of inherent phosphorylated Akt (p-Akt), however these levels were dramatically increased within 30 min of co-culture with either Bb or one of their prototypic lipoproteins, OspA (Figure 1A). While BMMs pretreated with LY had little effect on the background levels of p-Akt, this inhibitor completely suppressed the ability of both Bb and the OspA lipoprotein to produce p-Akt, indicating that these agonists activate the PI3 kinase pathway. To test whether IL-10 production in response to Bb and OspA are PI3 kinase-dependent, supernatants from BMMs that were pre-treated with or without LY prior to co-culture with these agonists were assessed by ELISA, and cellular transcript levels by qRT-PCR analyses. Unstimulated BMMs produced little to no background levels of any of the assessed cytokines, but co-culture with Bb caused a significant upregulation of all assessed cytokines, and addition of OspA and LPS also upregulated all mediators except IL-12 (Figure 1B). Pre-treatment with LY significantly reduced IL-10 production in response to Bb, as well as OspA and LPS down to baseline levels (Figure 1B). However, Bb elicitation of IL-12 was greatly enhanced by LY inhibition, whereas TNFα production
was reduced to baseline. Neither OspA nor LPS-stimulated cells produced IL-12 at detectible levels. Notably, Bb-elicitation of IL-6 was not affected by LY treatment, suggesting a difference in these signaling events from IL-10. Parallel experiments performed to assess LY effects on cytokine transcript levels showed a similar trend as those observed at the protein level, including an enhancing effect by LY on the production of the chemokine KC (Figure 1C). To confirm these data, parallel experiments were also performed using an additional PI3 kinase inhibitor, Wortmannin (10nM), and the results were consistent with those observed for LY (data not shown).

To further delineate the PI3 kinase-mediated pathways involved in Bb signaling, we examined the role of its downstream effector, mTOR, in Bb-induced IL-10 production. Immunoblot and desenitometry analysis demonstrated that unstimulated BMMs showed no activation of mTOR, as reflected by a lack of phosphorylation of the ribosomal protein p70/S6 (p-S6; Figure 2A), which is a standard method of assessing mTOR activity in mammalian cells (Weichhart, Costantino et al. 2008). Co-culture of BMMs with either Bb or OspA resulted in a significant increase in p-S6 levels, and the specificity of this response was tested in co-cultures pretreated with the specific inhibitor of mTOR, rapamycin, which substantially decreased the p-S6 levels produced in response to Bb and OspA. The importance of the mTOR pathway in the production of Bb-elicited IL-10 was assessed using rapamycin pre-treated co-cultures, which showed a significant suppression in the levels of IL-10 produced in response to Bb and OspA, while having little effect on production of IL-12 (Figure 2B). Taken together, these data indicate that Bb-elicitation of IL-10 occurs via the PI3 kinase/Akt/mTOR pathways, while most of the
proinflammatory mediators are produced independent of these pathways; TNFα appears to be the exception in that it is regulated like IL-10 in these experiments.

**Bb-elicitation of IL-10 by BMMs requires MAP kinase activity.** To determine whether signaling events involving MAP kinase family members were involved in the production of Bb-elicited IL-10, BMMs were co-cultured with Bb in the presence or absence of the MAP kinase p38 inhibitor SB203580 (SB) to determine the effects on phosphorylation of the p38 kinase (P-p38), which is indicative of activity (Lee, Kumar et al. 2000; Cuenda and Rousseau 2007). Immunoblot and densitometry analysis demonstrated that unstimulated BMMs contained no detectable P-p38, however these levels were dramatically increased within 30 min of co-culture with either Bb, OspA, or LPS (Figure 3A). While BMMs pretreated with SB had little effect on the background levels of P-p38, this inhibitor almost completely suppressed the ability of Bb, OspA, and LPS to induce appearance of P-p38, indicating that these agonists activate the p38 Map kinase pathway. To test whether IL-10 production in response to Bb and OspA are p38 MAP kinase-dependent, supernatants from BMMs that were pre-treated with or without SB prior to co-culture with these agonists were assessed by ELISA and cell lysates were harvested for qRT-PCR analyses. Unstimulated BMMs produced little to no background levels of any of the assessed cytokines, but stimulation with Bb caused a significant upregulation of all assessed cytokines, and addition of either OspA or LPS also upregulated all mediators (Figure 3B). Pre-treatment with SB significantly reduced IL-10 production in response to Bb, as well as reducing OspA and LPS responses to baseline levels (Figure 3B). However, Bb elicitation of IL-12 was enhanced by SB inhibition,
whereas TNFα production was reduced to baseline. Neither OspA nor LPS-stimulated cells produced significantly increased IL-12 levels. Notably, Bb-elicitation of IL-6 was not affected by SB treatment, again suggesting that differences exist in signaling events compared to IL-10 and TNFα. Parallel experiments performed to assess SB effects on cytokine transcript levels showed a similar trend as those observed at the protein level, including an enhancing effect by SB on the production of the chemokines KC (Figure 3C). To help confirm these data, parallel experiments were also performed using another p38 MAP kinase inhibitor, SB202190, and the results were consistent with those observed for SB203580 (data not shown).

To determine whether additional MAP kinase pathways were involved in Bb signaling, we examined the role of the ERK1/2 pathway in Bb-induced IL-10 production. Immunoblot and densitometry analysis demonstrated that unstimulated BMMs showed no activation of ERK1/2, as reflected by a lack of phosphorylation of the p40 and p42 subunits (p-ERK1/2; Figure 4A). Co-culture of BMMs with Bb, OspA, or LPS resulted in a significant increase in p-ERK1/2 levels, and the specificity of this response was tested in co-cultures pretreated with the ERK1/2-specific inhibitor U0126, which completely suppressed the p-ERK1/2 levels produced in response to Bb, OspA, and LPS. The importance of the ERK1/2 pathway in the production of Bb-elicited IL-10 was assessed using U0126 pre-treated co-cultures, which showed a significant suppression in the levels of IL-10 produced in response to Bb, OspA, and LPS (Figure 4B). Alternatively, the proinflammatory cytokines IL-12 and IL-6 were not suppressed in the presence of U0126, and in some cases were actually increased, whereas TNF was the exception in being ERK1/2 dependent, similar to IL-10. Taken together, these data
indicate that Bb-elicitation of IL-10 occurs via the PI3 kinase/Akt/mTOR pathways, while most of the proinflammatory mediators are produced independent of these pathways; TNFα appears to be the exception. Finally, parallel experiments performed to assess U0126 effects on cytokine transcript levels showed a similar trend as those observed at the protein level, where IL-10 transcripts were suppressed by U0126 but all inflammatory cytokines and chemokines were unaffected by this inhibitor (Figure 4C).

Taken together, these data indicate that Bb-elicitation of IL-10 is dependent on signaling through both the p38 and ERK1/2 MAP kinase pathways, while most of the proinflammatory mediators are produced independent of these pathways; TNFα again appears to be the exception.

**Bb-induced activation of PI3 kinase and MAP kinase pathways by BMMs are TLR2-dependent.** To determine whether TLR2 signaling is the upstream requirement of PI3 and MAP kinase activation in response to Bb and OspA, BMMs derived from both WT and TLR2−/− mice were compared for their abilities to activate these signaling pathways. While Bb and OspA were both able to upregulate these pathways in WT macrophages, as previously indicated (Figures 1-4), similar co-cultures containing TLR2−/− macrophages demonstrated either substantially lower or no phosphorylation of Akt, p38, or ERK1/2 in response to Bb and OspA as demonstrated by the immunoblot and densitometry analysis (Figure 5A). These low levels were not due to general signaling deficiencies in the TLR2−/− macrophages, as they were shown to robustly phosphorylate p38, and ERK in response to either LPS or intact *E. coli* (Figure 5B and data not shown).
These studies indicate that TLR2-mediated signaling events are necessary for appropriate activation of these key pathways in response to Bb stimulation.

**Bb-induced activation of PI3 kinase and MAP kinases by BMMs can occur in the absence of phagocytosis.** Since our studies indicated that the PI3 kinase and MAP kinases signaling were necessary for Bb elicitation of IL-10, we were also interested in determining if these pathways could be activated independent of phagocytosis. Immunoblot analyses again indicated that both Bb and OspA can substantially upregulate the PI3 kinase, p38, and ERK1/2 MAP kinase pathways within 30 min of activation (Figure 6A and B). Interestingly, with the exception of ERK1/2 activation in response to Bb, parallel experiments performed in the presence of CytoD demonstrated that macrophages produced similar or slightly enhanced activation of all three pathways compared to co-cultures that did not receive CytoD, indicating that activation of these pathways by Bb can occur independent of phagocytosis.

To address whether blocking these pathways under non-phagocytosis conditions would affect IL-10 production, ELISA analyses were performed on supernatants collected from parallel co-cultures. As shown previously, secretion of both IL-10 and IL-6 are strongly increased after exposure to Bb and OspA, and these levels are not changed if phagocytosis is blocked (Figure 6C). However, the addition of inhibitors that are specific for p38 and MAP kinase signaling cascades completely suppress the production of IL-10 in response to Bb and OspA, whereas IL-6 levels are unchanged. These findings indicate that signaling via these three pathways are critical for Bb-elicitation of IL-10 by
these early interactions with macrophage surface receptors, and thus may represent targets for blocking detrimental IL-10 responses.

**Blockage of p38 MAP kinase suppresses the production of Bb-elicited IL-10 in vivo:**

To address whether therapies directed towards MAP kinase pathways could block Bb-elicited IL-10 production in vivo, groups of mice were injected with the p38 MAP kinase inhibitor SB203580 (SB) and subsequently infected with Bb to assess the effects on cytokine production in skin tissues. Mice preinjected with PBS showed a strong upregulation of many cytokine transcripts, including IL-10, TNFα, and KC, in skin tissues within 24 h of infection (Figure 7). Mice preinjected with SB also upregulated the transcript levels of these cytokines after Bb infection, however the IL-10 transcripts levels were significantly less than in infected mice receiving PBS pretreatment, while the other transcript levels remained similar between the two groups. These findings suggest that treatments blocking the p38 MAP kinase pathways can block the detrimental production of IL-10 in response to Bb infection and might serve to enhance the immune responses to this pathogen during infection.

To assess whether the administration of p38 MAP kinase inhibitor to Bb-infected mice will promote enhanced immune clearance of Bb, groups of wt and IL-10⁻/⁻ mice were inoculated intradermally with Bb and the infection allowed to progress for three weeks, during which the mice were administered either the p38 inhibitor SB203580 or a PBS control every other day. After the three week infection, target tissues were harvested and the Bb levels were assessed. At three weeks post-infection, substantial Bb numbers are observed in all four of the assessed tissues from the PBS control treated
mice (Figure 8). Many of the tissues from the SB203580-treated mice showed altered Bb numbers compared to the PBS control, although only the skin tissue achieved statistical significance. Notably, the SB203580-treated tissues possessed Bb levels that were similar to those from IL-10−/− tissues, indicating the protective effects provided by p38-blockage were similar to blocking Bb-elicited IL-10 production. Tissues from the IL-10−/− mice treated with SB203580 showed no further reduction of Bb numbers compared to the control IL-10−/− mice, further suggesting that the protective effects provided by SB203580 treatment mirrors that provided by blocking the detrimental effects of IL-10. Additional experiments to increase the numbers of experimental values should further delineate the true protective effects of p38-based therapies and whether these effects are solely due to inhibition of IL-10 production.

**Discussion**

Lyme disease is largely defined by the inflammatory responses against the bacteria that are persisting and reemerging from different target tissues within the infected host. Therefore, there is much interest in defining the signaling pathways that are central to the development of Lyme disease. PI3 kinase and MAP kinases have been reported to be involved in host inflammatory responses against Bb (Ramesh and Philipp 2005; Hedrick, Olson et al. 2006; Olson, Hedrick et al. 2007; Sahay, Patsey et al. 2009; Shin, Miller et al. 2009). In addition, PI3 kinase and MAP kinases were reported as putative signaling cascades downstream of TLR2 that results in the production of IL-10 by BMMs in response to various agonists. (Shen, Kawamura et al.; Polumuri, Toshchakov et al. 2007; Shen, Kawamura et al. 2010). In these studies, we demonstrate
that IL-10 production by BMMs in response to Bb or its lipoprotein OspA requires both the signaling cascades involving PI3 kinase, as well as MAP kinases p38 and ERK1/2. The production of a small subset of pro-inflammatory cytokines, such as TNFα, was also dependent on these signaling components; a finding that is consistent with others (Yang, Zhang et al.; Chelvarajan, Popa et al. 2007). As for Bb-elicited IL-6, our previous study suggested that it shared signaling pathways with IL-10 production, where both appeared to be TLR2-dependent and signaling could occur independent of phagocytosis. However, our current studies have identified a unique feature between these pathways, in that IL-10 was dependent on signaling via PI3-kinase and the MAP kinase pathways, whereas IL-6 production could occur even when these pathways were inhibited. This pattern of IL-6 production by macrophages when the PI3-kinase and MAP kinase pathways were blocked has been observed by others as well (Sinsimer, Fallows et al. 2010) (Horwood, Page et al. 2006). Therefore, our findings suggest that the signaling cascades leading to production of Bb-elicited IL-10 and IL-6 by BMMs, although utilizing similar mechanisms that can proceed in the absence of phagocytosis, do possess clear differences with respect to intracellular signaling pathways.

Our findings indicate that Bb-elicited IL-12 displayed an opposite trend for dependence on both the PI3 kinase and the MAP kinases pathways compared to IL-10, where the inhibition of these signaling pathways enhanced IL-12 while suppressing IL-10 production. This reciprocal trend between IL-10 and IL-12 with respect to PI3 kinase and MAP kinase signaling has been observed by other researchers in response to a number of different pathogens or agonists (Kaji, Kiyoshima-Shibata et al.; Martin, Schifferle et al. 2003; Chelvarajan, Popa et al. 2007; Gunzl, Bauer et al. 2010; Yang,
Zhang et al. 2010). Since IL-10 is known to down-regulate IL-12 in Bb-stimulated BMMs, one possibility is that the upregulation of IL-12 seen in Bb-stimulated BMMs in the presence of PI3 kinase or MAP kinase inhibitors was purely indirect, due to the down-regulation of IL-10. To address this issue, parallel studies either using BMMs from IL-10−/− mice or in the presence of neutralizing IL-10 antibodies were performed, and in either case no differences in IL-12 production were observed with or without PI3 kinase or MAP kinase inhibitors (data not shown). Direct evidence have also been reported that link PI3 kinase activation to the suppression of IL-12 production in BMMs (Zhou, Collins et al. 2010). Stimulation of BMMs from neonates with LPS has been reported to upregulate IL-12 in the absence of MAP kinase signaling, and appears independent of the down-regulation of IL-10 (Chelvarajan, Popa et al. 2007). Studies involving *Lactobacteria spps* indicated that bacterial cell wall components, such as teichoic acid, can modulate the production of either IL-10 or IL-12 from macrophages in a MAP kinases-dependent matter (Kaji, Kiyoshima-Shibata et al.). Although our data suggests that Bb-stimulated BMMs display a similar cytokine production pattern as *Lactobacteria spps*, Bb is not known to express teichoic acid and Bb lipoproteins elicit very little IL-12, although both are reported to signal via TLR2. This indicates there may exist unidentified structural componenst of Bb that are responsible for the MAP kinase-regulated production of IL-10 and IL-12 in host BMMs. Overall, the reciprocal pattern of Bb-elicited IL-10 and IL-12 production, with respect to PI3 kinase and MAP kinase pathways, may warrant the design of pharmacological therapeutics to promote resolution of Lyme disease.
Our *in vivo* data demonstrated that Bb-infected mice receiving sub-cutaneous injection of the MAP kinase p38 inhibitor SB203580 produced significantly lower IL-10 transcript levels as compared to mice receiving control injections, while not adversely affecting TNFα or KC levels, which was consistent with our *in vitro* BMM studies. Because skin tissues contain many different cell types that are capable of producing IL-10 in response to Bb infections (e.g. keratinocytes, Langerhans cells, etc.), the involvement of MAP kinase in the production of IL-10 may not be limited to BMMs alone. Our *in vivo* findings are consistent with others that utilized *in vivo* administration of various MAP kinase inhibitors to attenuate Leishmania-induced IL-10 levels in footpads (Yang, Mosser et al. 2007; Yang, Zhang et al. 2010); further suggesting that therapies introducing MAP kinase inhibitors can be an effective mechanism for attenuating pathogen-induced IL-10 production *in vivo*, and that other cell types besides BMMs may be affected by these IL-10-suppressive therapies. Although we were not able to detect IL-12 levels in the skin of Bb-infected animals, inhibitors of p38 MAP kinase such as SB203580 were previously reported to down-regulate serum IL-12 levels in response to Bb infection, which is somewhat inconsistent with our *in vitro* Bb-stimulated BMM studies (Hedrick, Olson et al. 2006). One possibility is that MAP kinase activation in other cells may not have the same effects as those for BMMs or other APCs in term of IL-12 production. Another possibility is that SB injection, due to its ability to attenuate IL-10, resulted in better clearance of Bb by innate immunity, resulting in a lower Bb load and less exposure of Bb agonist to the responding immune cells. Our current studies attempt to address these issues by performing long-term Bb infection studies in mice receiving MAP kinase inhibitor every two days, in order to better
correlate these effects on suppressing IL-10 production to the overall enhancement of Bb clearance. These preliminary findings suggest that administration of the p38 MAP kinase inhibitor SB203580 \textit{in-vivo} may reduce Bb levels within the skin and other tissues down to the levels typically seen in IL-10 \textsuperscript{-/-} mice. This finding is consistent with the finding that the suppression of MAP kinase signaling leads to reduced levels of IL-10 within the skin while leaving pro-inflammatory cytokines (e.g. KC) unaffected. This finding suggests that administration of p38 MAP kinase inhibitors may therapeutically reduce Bb levels during the early course of infection. However, more detailed studies are needed to confirm if these reductions in Bb numbers are repeatable and significant. If further studies find that no significant differences are attained, there are a few possible explanations for this lack of effect. First, p38 MAP kinase may not be the global signaling pathways utilized by the cells important for IL-10 production in response to Bb. Therefore, IL-10 producing cells outside of the skin may not be affected by the p38 inhibitors as much as those residing within the skin. This would still allow IL-10 production to persist once Bb leaves the skin and migrates toward target tissues. Another possibility is that the p38 inhibitors may inhibit critical host defense components along with IL-10 production in tissues outside of skin, which counter-balances the effect of Bb clearance by the suppression of IL-10. As aforementioned, the results obtained in Figure 8 could be a reflection of a lack of sample size, so repeating this experiment in repetition would help to increase the mouse numbers as well as to validate the findings.

Our studies also suggest that activation of both PI3 kinase and various MAP kinases by BMMs in response to Bb and/or its lipoproteins occur downstream of TLR2, and are both independent of Bb phagocytosis, which allows us to model the putative
signaling pathways leading to the production of Bb-elicited IL-10 by BMMs (Figure 8). Previous studies have demonstrated that the PI3 kinase and MAP kinase signaling cascades can either reciprocally suppress one another (Gunzl and Schabbauer 2008; Luyendyk, Schabbauer et al. 2008) or be completely independent of each other (Mendez-Samperio, Trejo et al. 2008). When we inhibited PI3 kinase, we observed only minimal effects on the phosphorylation of p38 MAP kinase in response to Bb and OspA, and vice-versa (data not shown). Therefore, despite any similarities in the involvement of PI3 kinase and the MAP kinase pathways in cytokine production in response to Bb, there is little evidence supporting the two pathways cross-talk. However, evidence suggests that both PI3 kinase and MAP kinases activation within macrophages do share some common upstream effectors. One possibility is the spleen tyrosine kinase (SYK), which has been reported to be involved in myeloid leukocyte signaling downstream of TLRs (Mocsai, Ruland et al. 2010). In fact, SYK has been implicated in the ERK1/2-dependent production of IL-10 by macrophages in response to Leishmania spps (Yang, Mosser et al. 2007). Besides common upstream factors, both PI3 kinase and MAP kinases can share putative downstream signaling pathways for IL-10 production as well. For example, NF-κB has been demonstrated to be regulated by both PI3 and MAP kinase pathways in immune cells, and has been reported to be involved in inflammatory signaling in response to agonists associated with Bb (Wooten, Modur et al. 1996; Xie, Patel et al. 2007; Sadik, Hunfeld et al. 2008). Additional studies are needed to further delineate the signaling cascades that activate the PI3 kinase and MAP kinases pathways in response to Bb, the receptors that initiate these signals, and which pathways are critical for Bb-elicitation of IL-10.
Figure 1

A

\[
\begin{array}{cccccccc}
\text{Unst} & \text{Bb} & \text{OspA} & \text{Unst} & \text{Bb} & \text{OspA} \\
p-Akt & & & & & & \\
actin & & & & & & \\
\hline \\
LY294003 & - & - & - & + & + & + \\
\end{array}
\]

\[\text{pAKT/actin} \]

B

\[
\begin{array}{cccc}
\text{Unstimulated} & \text{Bb} & \text{OspA} & \text{LPS} \\
\text{IL-10} & \text{IL-12} & \text{TNF}_{\alpha} & \text{IL-6} \\
\text{ng/ml IL-10} & \text{ng/ml IL-12} & \text{ng/ml TNF}_{\alpha} & \text{ng/ml IL-6} \\
\text{Veh} & \text{LY294002} & \text{Veh} & \text{LY294002} \\
\end{array}
\]

\[\text{*} \]
Figure 1: Production of IL-10 by BMMs in response to Bb is PI3 kinase dependent.

Cultured BMMs were pre-treated with vehicle (DMSO) or 50µM of LY294002 (specific inhibitor of PI3-kinase) for 1h prior to stimulation with Bb (MOI=10), OspA (100ng/ml), or LPS (1μg/ml). (A), Top: Levels of phosphorylated-Akt were assessed at 30 minutes post-stimulation by immunoblot. Bottom: Quantitative densitometry analysis was performed to determine relative levels of phosphorylated-Akt over actin for each sample of the representative immunoblot shown. (B), Cytokine levels were assessed in co-culture supernatants after 24h stimulation via ELISA. (C), Transcript levels of cytokines/chemokines in BMMs at 8h post-stimulation were assessed via real-time qPCR. Data is expressed as percent changes in transcript levels from Bb-stimulated DMSO-treated BMMs and is shown as the average of at least three separate experiments. (* indicates P< 0.05 compared to DMSO-treated BMMs)
Figure 2: Production of IL-10 by BMMs in response to Bb involves mTOR. BMMs were pre-treated with DMSO vehicle or 25 nM of rapamycin (specific inhibitor of mTOR) for 1h prior to stimulation with Bb (MOI=10) or OspA (100ng/ml). (A), Top: Assessment of S6 phosphorylation in BMMs at 30 min post-stimulation by immunoblot. Bottom: Quantitative densitometry analysis was performed to determine relative levels of phosphorylated-S6 normalized to total S6 for the representative immunoblot shown. (B), ELISA analyses of IL-10 and IL-12 in co-culture supernatants at 24h post-stimulation. Data shown is representative of at least three separate experiments. (* indicates P< 0.05 compared to vehicle control)
Figure 3

A

B

Figure 3

A

B

Figure 3

A

B
Figure 3: Production of IL-10 by BMMs in response to Bb is MAP kinase p38-dependent. BMMs were treated with vehicle (DMSO) or 10µM of SB203580 (specific inhibitor of MAP kinase p38) for one hour prior to stimulation with Bb, OspA, or LPS. (A), Top: Levels of phosphorylated p38 were assessed after 30 minutes co-culture by immunoblot. Bottom: Quantitative densitometry analysis was performed to determine relative levels of phosphorylated-p38 normalized to actin for each sample of the representative blot shown. (B), Cytokine production was assessed at 24 h post-stimulation by ELISA. (C), Transcript levels of cytokines/chemokines in BMMs were assessed at 8h post-stimulation via real-time qPCR. Data is expressed as percent changes in transcript levels from Bb-stimulated DMSO-treated BMMs and is shown as the average of at least three separate experiments. (* indicates P< 0.05 compared to DMSO-treated BMMs)
Figure 4

A

![Graph showing p-ERK1/2 and actin levels with different treatments and U0126 concentrations.]

B

![Graph showing cytokine levels (IL-10, IL-12, TNFα, IL-6) with different treatments and U0126 concentrations.]

Legend:

- Unstimulated
- Bb
- OspA
- LPS

* Denotes statistically significant differences.
Figure 4: Production of IL-10 by BMMs in response to Bb is MAP kinase ERK1/2-dependent. BMMs were treated with vehicle (DMSO) or the indicated doses of U0126 (specific inhibitor of MAP kinase ERK1/2) for one hour prior to stimulation with Bb, OspA, or LPS. (A), Top: Levels of phosphorylated ERK1/2 were assessed at 30 minutes post-stimulation. Bottom: Quantitative densitometry analysis was performed to determine relative levels of phosphorylated-pERK1/2 normalized to actin for each sample of the representative immunoblot shown. (B), BMMs were pretreated with 10µM U0126 for one hour prior to stimulation and cytokine levels were assessed at 24h post-stimulation via ELISA. (C), Transcript levels of cytokines/chemokines in BMMs at 8 h post-stimulation via real-time qPCR. Data is expressed as percent changes in transcript levels from Bb-stimulated DMSO-treated BMMs and is shown as the average of at least three separate experiments. (* indicates P< 0.05 compared to DMSO-treated BMMs)
Figure 5

A

**PI3 Kinase/Akt**

<table>
<thead>
<tr>
<th></th>
<th>Unst</th>
<th>Bb</th>
<th>OspA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p-Akt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Akt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MAP Kinase p38**

<table>
<thead>
<tr>
<th></th>
<th>Unst</th>
<th>Bb</th>
<th>OspA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p-p38</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Histone H3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**WT** vs. **TLR2−/−**: Comparison of PI3 Kinase/Akt and MAP Kinase p38 levels in Unst, Bb, and OspA conditions.
MAP Kinase ERK1/2

WT

TLR2⁻/⁻

p-ERK1/2
Histone H3

WT

TLR2⁻/⁻

p-p38
H3

B
Figure 5: Activation of PI3 kinase and MAP kinase pathways by BMMs in response to Bb and its lipoproteins are TLR2-dependent. (A) BMMs isolated from WT or TLR2−/− mice were stimulated with either Bb or OspA for 30 minutes before assessing for phosphorylated forms of Akt, p38, or ERK1/2. (B) BMMs from WT and TLR2−/− mice were stimulated with LPS at 1μg/ml and blotted for the phosphorylated form of p38 as positive control for cell stimulation. Quantitative densitometry analysis was performed for all blots to determine relative levels of phosphorylated-proteins normalized to either the total protein levels or histone H3 for the blot shown.
Figure 6

A

CytoD | Unst | Bb | OspA
--|---|---|---
- | - | + | +
- | + | - | -
- | + | - | -

Phospho-Akt
Phospho-p38
Actin

B

Cyto D | Unst | Bb | OspA
--|---|---|---
- | - | - | -
+ | - | - | -
+ | - | - | -

Phospho-ERK1/2
Histone H3

p-ERK/Actin

0 1 2 3
- | - | - | -
+ | - | - | -
+ | - | - | -
Figure 6: Bb activation of PI3 kinase and MAP kinase pathways that lead to IL-10 production by BMMs occurs independent of Bb phagocytosis. BMMs were treated with Cyto D (2 μM) for one hour prior to stimulation with Bb or OspA, and co-cultures were assessed for phosphorylation of (A) Akt, p38, and (B) ERK1/2 at 30 min post-stimulation by immunoblot. (C), BMMs were co-treated with CytoD (2 μM) along with either LY294002 (50 μM), SB203580 (10 μM), or U0126 (10 μM) for one hour prior to overnight stimulation with Bb, OspA, or LPS. Production of IL-10 and IL-6 were assessed in culture supernatants via ELISA (* indicates P<0.05 when DMSO treated cells are compared to those receiving LY, SB or U. ** indicates P<0.05 when non-CytoD treated cells are compared to those receiving CytoD).
Figure 7: *In vivo* effect of MAP kinase inhibitor on cytokine production in Bb-infected skin tissues. Mice were pre-injected sub-cutaneously with either PBS or SB203580 for one hour prior to intra-dermal inoculation with $10^4$ Bb. Skin tissues were harvested from the Bb-inoculation site 30h later, total RNA from these tissues was reverse transcribed, and cytokine levels (IL-10, TNF, and KC) were assessed by qRT-PCR. Values shown indicate fold-increases for each cytokine transcript levels compared to BSK-inoculated skin. Each point represents an individual mouse tissue (* indicates $P < 0.05$ compared to PBS control).
Figure 8

A

Heart

Skin

○ wt

○ IL-10 KO

Left Ankle

Left Ear
B

Average values of Bb genome/1000 mouse genomes from harvested tissues for each treatment group

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>wt</th>
<th>IL-10 -/-</th>
<th>IL-10 -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>SB203580</td>
<td>PBS</td>
<td>SB203580</td>
</tr>
<tr>
<td>Heart</td>
<td>5.4</td>
<td>2.7</td>
<td>*0.8</td>
<td>*1.7</td>
</tr>
<tr>
<td>Skin</td>
<td>93.3</td>
<td>*2.7</td>
<td>*2.6</td>
<td>*3.5</td>
</tr>
<tr>
<td>Ankle</td>
<td>5.6</td>
<td>2.0</td>
<td>*1.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Ear</td>
<td>3.2</td>
<td>3.5</td>
<td>1.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Figure 8: *In vivo* effect of p38 MAP kinase inhibition on Bb clearance. Groups of wt and IL-10 /- strains were inoculated intradermally with 2x10^4 Bb. Mice from each strain were further divided into two groups containing 7-8 mice per group. To one group, the p38 MAP kinase inhibitor SB203580 (SB; 5mg/kg animal weight) was administered on the day of infection and then every other day for up to three weeks, while the other received PBS throughout the same time as control. Mice were euthanized after three weeks of Bb infection, when the heart, skin from the back (distant from injection site in ear), the entire ear, and the ankle were harvested for DNA purification, and Bb levels within these tissues were assessed using quantitative PCR. (A), Bb load per tissue plotted as number of Bb genomes (*recA*) normalized per 1000 host genomes (*nidogen*) for each groups (black = wt, blue = IL-10 /-). Each circle represents the value from an individual mouse. (B), Average values of Bb load per tissue as indicated by the horizontal line drawn in (A) (* indicates P<0.05 compared to Bb-infected wt PBS control).
Figure 9: Putative intracellular signaling pathways in BMMs important for the production of Bb-elicited IL-10. Our current model indicates that the mechanisms involved in the production of Bb-elicited IL-10 by macrophages are initiated by surface interactions between either viable Bb or Bb lipoproteins and TLR2. This TLR2-mediated interaction leads to activation of PI3-kinase/Akt signaling pathway, and the subsequent activation of mTOR pathways. Similarly, Bb also induces activation of MAP kinase pathways p38 and ERK1/2 downstream of surface TLR2 interaction with Bb, leading to the production of Bb-elicited IL-10.
Chapter 5

Discussions and Novel Findings

Overview of major goals:

The bacterium *Borrelia burgdorferi* (Bb) is an obligate host parasite which requires it to dwell within an immunocompetent animal host, as it lacks several important genes for synthesizing essential nutrients and key structural components (Casjens, Palmer et al. 2000; Tilly, Rosa et al. 2008). Because of this, Bb has adapted many strategies to evade host immune mediators, allowing it to persist long term in susceptible hosts (Fikrig and Narasimhan 2006; Tilly, Rosa et al. 2008). As a result, Bb infection will often elicit strong inflammatory and adaptive immune responses, but still have incomplete clearance of the bacteria, leading to the persistence of Bb and development of Lyme disease (LD). Many of the described immune-evasive strategies employed by Bb were achieved through the variable expression patterns of its surface lipoproteins (i.e. antigen variation)(Singh and Girschick 2004). However, there is still much to understand regarding how Bb can survive the multi-component immune activation by the infected host and achieve persistence.
Recent studies have revealed that the anti-inflammatory cytokine IL-10 plays a significant suppressive role in the clearance of Bb. Bb-infected IL-10−/− mice display significantly reduced bacterial loads in tissues, as well as an eight-fold greater ID50 for Bb compared to wild-type mice (Brown, Zachary et al. 1999; Lazarus, Meadows et al. 2006). High levels of IL-10 transcripts are detectible in host skin tissue near the injection site within 24 hours of infection (Lazarus, Kay et al. 2008). In vitro studies indicated that both intact Bb and recombinant Bb lipoproteins can rapidly elicit IL-10 production in macrophages. The levels of IL-10 produced by macrophages in response to Bb is capable of suppressing naïve macrophages from subsequent stimulation (Lazarus, Kay et al. 2008). These findings suggest that Bb rapidly elicits IL-10 production from host innate immune cells upon infection and that the immune suppressive properties related to these secreted levels help promote escape from immune clearance.

Much is still unknown regarding the precise mechanisms involved in IL-10 production by host cells in response to Bb and its effects on immune clearance. For these dissertation studies, I will attempt to address three important issues: 1) the effect of Bb-elicited IL-10 on the immune mediators elicited by resident antigen-presenting cells (APC), such as macrophages and dendritic cells (DC), 2) the critical surface interactions between Bb and macrophages (i.e. the sensing of Bb by surface receptors) involved in the production of Bb-elicited IL-10, and 3) the critical intracellular signaling pathways involved in the production of Bb-elicited IL-10 by macrophages. In the following sections, I will describe the key findings from each chapter and discuss some of the important implications.
**Both macrophages and dendritic cells produce high levels of IL-10 in response to viable Bb:** It was demonstrated in these studies that when both cultured macrophages and dendritic cells were stimulated with viable Bb, the production of IL-10 is detectible in supernatant as early as 4-8 hours post-stimulation and continue to rise up to 48 hours. While the kinetics of IL-10 production observed in macrophages is consistent with our previous findings (Lazarus, Meadows et al. 2006; Lazarus, Kay et al. 2008), here we also demonstrate that Bb-stimulated dendritic cells produce IL-10 in a similar fashion. This indicated that other innate immune cells besides macrophages are also capable of producing IL-10 in response to Bb infection.

Resident APCs such as macrophages and DCs have been known to produce IL-10 in response to various agonists (Moore, de Waal Malefyt et al. 2001). For example, macrophages were found to be major producers of IL-10 that were elicited by pathogens such as *Leishmania spps* (Yang, Mosser et al. 2007) and *Yersinia spps* (Sing, Roggenkamp et al. 2002). Myeloid derived DCs were known to be the most potent IL-10 producers among other subsets of DC (Boonstra, Rajsbaum et al. 2006) and can serve as sources of host IL-10 elicited by *Bordatella spps* (Nagamatsu, Kuwae et al. 2009). Although APCs are important in initiating immune responses against invading pathogens, they can also be manipulated by pathogens to produce dysregulated levels of IL-10 to suppress these responses. Reasons why APCs are prime targets for pathogen-mediated IL-10 are that 1) they expresses an abundant amount of toll-like receptors (TLRs) that are important for the production of both IL-10 and pro-inflammatory mediators, and 2) the abundant levels of IL-10 receptors present on the surface of APCs allow both a rapid response to IL-10 and further propagation of IL-10 production, since positive feedback of
IL-10 production through the signaling of IL-10 receptors has been shown (Mosser and Zhang 2008). Therefore, the manipulation of APC functions by invading microbes is essential for their persistance within immuno-competent hosts.

There currently are many studies intended to understand the precise mechanisms behind how different pathogens elicit IL-10 from resident APCs. As for Bb, my thesis studies indicate that the production of IL-10 in response to Bb requires surface TLR2 and the subsequent activation of PI3 kinase and/or MAP kinase pathways (to be discussed later) by macrophages. However, whether similar mechanisms exist within dendritic cells has yet to be studied. Studies from *H. pylori* infection have indicated that TLR2 expressed on DCs were required for the production of IL-10 in response to the bacterium (Wang, Gorvel et al. 2010). Therefore, it is likely that common mechanisms involved in pathogen-induced IL-10 production exist among immune cells.

Our current findings imply that DCs, along with macrophages, contribute to the production of IL-10 previously observed in murine skin within 24 hours of Bb-infection (Lazarus, Kay et al. 2008). However, which of the two APCs are more important as the source for Bb-elicited IL-10 within the skin is yet to be determined. Besides APCs, there are other putative IL-10 producing leukocytes within skin during early stages of Bb-infection. One is the Langerhan cells (LCs), a subset of dendritic cells that reside within the epidermis of the skin. LCs are known to express high levels of toll-like receptors on their surface, as well as being potent producers of IL-10 in response to TLR2 agonists (Flacher, Bouschbacher et al. 2006). Non-APC leukocytes, such as neutrophils, have also been found to be sources of IL-10 production, particularly during insults to the skin (Noel, Wang et al.)2011). Besides the immune cells, other cells such as keratinocytes,
which have been shown to be actively involved in IL-10 production (Byun, Choi et al. 2009), may also contribute to the source of Bb-elicited IL-10. Taken together, additional studies are required to delineate the major cell type(s) that are involved in IL-10 production during the early stages of Bb infection.

**Bb-elicited IL-10 subsequently suppresses various APC immune effectors against Bb:** Major findings from this thesis work indicates that various innate immune responses from both macrophages and dendritic cells directed against Bb can be suppressed by the levels of host IL-10 elicited by Bb. One of these down-regulated responses is the production of neutrophil-recruiting chemokines such as KC and MIP-2. This implies a reduced number and impaired activities of neutrophils at the site of Bb infection as a result of the elicited IL-10. Studies have implicated that an upregulation of KC in the skin after Bb infection leads to an increase of neutrophil influx, which subsequently leads to an enhancement of Bb-clearance (Xu, Seemanapalli et al. 2007). There have also been reports showing that *in-vitro*, Bb are susceptible to many immune mediators elicited by activated neutrophils (*e.g.* elastase and ROS) (Lusitani, Malawista et al. 2002). In fact, neutrophils were known to be one of the principal cells regulated by IL-10, due to the high levels of IL-10 receptors present on their cell surface (Tamassia, Calzetti et al. 2008). Therefore, investigating roles of IL-10 in neutrophil influx and activation in response to Bb is important. Intravital imaging studies using mice that express fluorescent neutrophil markers (*e.g.* LysM) crossed to IL-10−/− mice should provide a powerful tool to analyze neutrophils influx into Bb-infected tissues with respect to IL-10 *in vivo*. In addition to neutrophil-recruiting chemokines, Bb-elicited IL-10 also
suppresses mononuclear cell-recruiting chemokines such as MIP1α. This implies an overall impaired migration of leukocytes toward region of Bb-infection due to the dysregulated levels of IL-10.

The suppression of phagocytosis and respiratory burst by macrophages in response to Bb-elicited IL-10 suggests that this anti-inflammatory cytokine can directly depress the ability of phagocytes to kill internalized Bb. The role of IL-10 in the hindering of phagocytosis and intracellular trafficking of microbes by APCs is relatively novel. However, the ability of IL-10 to inhibit the NADPH oxidase assembly, the principle enzyme involved in the respiratory burst, has been reported (Qian, Hong et al. 2006). Since reactive oxygen species have been shown to damage Bb and their lipoproteins (Boylan, Lawrence et al. 2008), the suppression of respiratory burst by IL-10 may prolong their survival within phagolysosomes, thus increasing their chance of escaping the macrophages. However, assays that can assess Bb viability within phagocytes are currently unavailable. Therefore, further advancements on understanding the interactions between Bb and phagolysosome of macrophages will be challenging.

The role of ROS in immune clearance of Bb is currently controversial. While some studies report that ROS severely damages Bb surface lipoproteins and that antioxidant enzymes such as superoxide dismutase is required in order for Bb to survive environments rich in ROS (Boylan and Gherardini 2008; Boylan, Lawrence et al. 2008; Esteve-Gassent, Elliott et al. 2009), others showed no significant differences in Bb clearance between WT and NADPH oxidase-deficient mice (Crandall, Ma et al. 2005). One possible way to explain this discrepancy is that the elicited IL-10 suppressed ROS below the levels necessary for Bb-clearance. To determine whether differences in the
overall ROS production exist between Bb-infected WT and IL-10−/− mice, studies using
in-vivo chemiluminescence are needed. Interestingly, our studies determined that dendritic cells elicited very low to no respiratory burst in response to Bb as compared to macrophages. However, other studies have shown that DCs are capable of producing ROS when primed with certain agonists, such as LPS or CD40L (Vulcano, Dusi et al. 2004). Therefore, Bb may not possess the proper agonists for stimulation of ROS production by DCs.

One function of APCs is to activate T cells through the secretion of IL-12 and upregulation of surface co-stimulatory molecules, such as CD80, CD86, and CD40, as well as present antigens on MHC complexes. Our current findings indicate that IL-10 suppresses the upregulation of CD86 surface expression by APCs in response to Bb. Recently, it has been reported that within DCs, IL-10 upregulates the activity of the ubiquitin E3 ligase MARCH-1 to target both MHCII and CD86 for proteasome degradation (Jabbour, Campbell et al. 2009; Tze, Horikawa et al. 2011). This mechanism would partially explain the inherent higher levels of CD86 on APCs from IL-10−/− mice over WT mice, as seen in Figure 7 of Chapter 2. However, I failed to see a difference in effect of IL-10 on MHCII expression on APCs, suggesting that alternative mechanisms of IL-10 activity may exist for modulating co-stimulatory molecules on APCs in response to Bb.

The suppression of Bb-elicited IL-12 and co-stimulatory markers such as CD86 by IL-10 could explain previous studies reporting that T and B lymphocytes played small roles in Bb clearance (Barthold and Bockenstedt 1993; de Souza, Smith et al. 1993). Although there are limited findings on the direct effects of IL-10 on adaptive immune
response against Bb, our group has indicated a significantly greater level of Bb-specific antibodies present in the serum of Bb-infected IL-10−/− mice versus those of wt (Lazarus, Meadows et al. 2006). The fact that CD86 upregulation from APCs in response to Bb is suppressible by IL-10 further suggests that Bb can suppress T cell activation through the activities of host IL-10. Studies showed that the injection of blocking antibodies against CD86, but not CD80, alters Bb-mediated Th1/Th2 cytokine productions in-vivo (Shanafelt, Kang et al. 1998). Many long-term parasites have evolved strategies to suppress activation of adaptive immune responses by APCs. For example, the nematode *Heligmosomoides polygyrus* secretes molecules that suppresses the ability of dendritic cells to activate specific T cells against the pathogen (Segura, Su et al. 2007). Whether or not Bb utilizes a similar strategy by the elicitation of host IL-10 remained to be identified.

One conclusion from our studies is that both bone-marrow derived macrophages and DCs elicit similar immune responses when stimulated by Bb. Both cell types produce high levels of IL-10 and other cytokines/chemokines such as IL-6, IL-12, KC, and MIP-2 in response to Bb. In addition, both cell types readily upregulates surface CD86 during Bb-stimulation. Both cell types also respond similarly to IL-10 in the suppression of immune effectors against Bb. However, there are also differences in how these two cell types respond to Bb. As aforementioned, macrophages produce substantially greater levels of ROS compared to DCs when stimulated with Bb. Besides ROS, there were neither detectible levels of upregulation of either NO (Chapter 2, Figure 6) nor TNFα (data not shown) by DCs when stimulated with Bb as compared to macrophages. Although the DCs seen within my studies were not able to produce these immune effectors in response to Bb, there exists a subset of DCs, known as TNFα-iNOS
producing DCs (TIP-DCs), that could respond to pathogens by producing high levels of NO and TNFα, as well as some other functions similar to inflammatory macrophages (Serbina, Salazar-Mather et al. 2003). Although TIP-DCs originate from bone marrow, the mature form is often found in the spleen and liver. Studies implicated that intracellular pathogens such as *Listeria monocytogenes* or protozoan parasites such as *Trypanosomal brucei* are capable of differentiating bone-marrow DCs into TIP-DCs (Serbina, Salazar-Mather et al. 2003; Bosschaerts, Guilliams et al. 2010). Importantly, TIP-DCs are known as potent responders to IL-10 (Guilliams, Movahedi et al. 2009). Currently, there have been no reports on the role of TIP-DCs in Bb infection. Nevertheless, it would be beneficial to understand how other subsets of DC interact with Bb and their role in the clearance of the pathogen.

**The production of Bb-elicited IL-10 by macrophages is independent of Bb phagocytosis yet partially dependent on TLR2:** In order for Bb-elicited IL-10 to exert optimal inhibition of immune mechanisms important for Bb-clearance, it must be produced at a more efficient rate than those of the pro-inflammatory responses. Major findings from our previous studies (Lazarus, Meadows et al. 2006; Lazarus, Kay et al. 2008) as well as Chapter 2 of my thesis work indicate that the production of IL-10 by culture APCs in response to Bb can occur at around 4-6 hours post-stimulation. This is usually the time frame for the production of early pro-inflammatory cytokines such as TNF and IL-6 by macrophages. To better understand the mechanisms behind this rapid rate of Bb-elicited IL-10 production by innate immune cells, the role of phagocytosis and toll-like receptor 2 (TLR2), two early mechanisms involved in the sensing of microbial...
pathogens by macrophages (Underhill and Ozinsky 2002), were examined in the context of the production of IL-10 and pro-inflammatory mediators. When cytochalasin D (CytoD), an inhibitor of F-actin polymerization, was used to inhibit the phagocytosis of Bb by macrophages, the production of most pro-inflammatory mediators, such as IL-12 and TNFα, were inhibited. On the other hand, the production of IL-10 and IL-6 remained unchanged. Phagocytosis has been demonstrated to be important for macrophages to process internalized pathogens and to transduce signals important for the activation of inflammatory responses (Underhill and Ozinsky 2002; Akira 2006). Studies indicated that the elicitation of host pro-inflammatory cytokines production from phagocytes by various pathogens such as C. albican, H. pylori, and S. aureus were also phagocytosis-dependent (Chiani, Bromuro et al. 2000; Kranzer, Sollner et al. 2005; Ip, Sokolovska et al. 2010). As for Bb, previous literature suggests that production of Bb-elicited pro-inflammatory cytokines such as TNFα requires the intake of the bacteria by macrophages (Moore, Cruz et al. 2007; Shin, Isberg et al. 2008), which is consistent with our findings. In addition to the above findings, we have identified that non-cytokine immune effectors, such as nitric oxide, respiratory burst, and upregulation of surface CD86 in response to Bb were also phagocytosis-dependent. Therefore, the phagocytosis and internalization of Bb by macrophages is required for the optimal propagation of pro-inflammatory responses against the bacterium.

Unlike pro-inflammatory responses, the elicitation of IL-10 production by macrophages in response to Bb is independent of phagocytosis/Bb intake. This argues for the first time that distinct pathways may exist between productions of IL-10 versus pro-inflammatory mediators by macrophages in response to Bb. It has been reported that
the Gram-negative bacteria *Helicobacter pylori* can also elicit IL-10 and IL-6 from APCs independent of phagocytosis (Kranzer, Sollner et al. 2005). In Chapter 3, comparative studies performed using *E. coli* indicate that IL-10 and IL-6 elicitation in response to *E. coli* was likewise independent of phagocytosis. Therefore, the ability to induce host IL-10 or IL-6 production by APCs through only surface interactions as seen by Bb could be a common property among Gram-negative bacteria. One possibility can be linked to the fact that prototypical Gram-negative bacteria contain an outer membrane that is rich in agonists that are capable of interacting with multiple surface TLRs. Chapter 3 also demonstrated that *E. coli* is still capable of eliciting some pro-inflammatory cytokine in the absence of phagocytosis, whereas for Bb, the elicitation of nearly all pro-inflammatory responses requires the internalization of the bacteria. Since phagocytosis and phagosome maturation are energy-consuming processes, Bb-elicited IL-10 is expected to be produced at a faster and more efficient rate than pro-inflammatory responses by macrophages, thus making the elicitation of host IL-10 a preferred mechanism for Bb to evade host immune clearance.

TLR2 has been reported to recognize the triacylated Bb lipoproteins as well as intact Bb (Hirschfeld, Kirschning et al. 1999; Shin, Isberg et al. 2008), and is involved in the induction of IL-10 production by macrophages in response to Bb-lipoproteins (Wooten, Ma et al. 2002). Therefore, TLR2 expressed on the surface of mammalian cells along with the lipoproteins on the surface of Bb may be the principle receptor and ligand involved in elicitation of host IL-10. The findings in Chapter 3 showed the production of IL-10 by macrophages in response to intact Bb are partially-dependent on surface TLR2-mediated events, whereas IL-10 production in response to Bb surface lipoprotein OspA is
completely-dependent on surface TLR2 signaling. Chapter 4 showed that the elicitation of IL-10 by Bb and OspA shares many common intracellular signaling cascades such as PI3-kinase and MAP kinase pathways. These findings support that host TLR2 and Bb surface lipoproteins are important for the elicitation of host IL-10 production. However, neither TLR2 nor Bb lipoprotein alone may be sufficient for the production of IL-10 by macrophages in response to Bb. Although OspA contains the immunogenic components important for TLR2 signaling (i.e. the tri-acylated lipids) that is found on all Bb lipoproteins, there are at least 130 different putative lipoproteins expressed on the surface of Bb. Therefore, a proteomic analysis of Bb surface components is needed to potentially identify motifs important for host IL-10 elicitation. Similarly, there is a need to identify additional mammalian cell surface receptors other than TLR2 that may be important for the production of Bb-elicited IL-10. For example, studies indicated that TLR2 tends to associate with other surface receptors/molecules, such as TLR1 or TLR6 (Alexopoulou, Thomas et al. 2002). Studies also indicate that in the absence of MyD88, an intracellular adapter protein associated with the function of various surface TLRs, the production of cytokines by macrophages in response to intact Bb were significantly lower than the loss of an individual TLR (such as TLR2) (Shin, Isberg et al. 2008; Dennis, Dixit et al. 2009). Recent studies also indicated that the association of TLR2 along with intracellular TLRs such as TLR8 is required for the optimal production of cytokines by macrophages in response to Bb (Cervantes, Dunham-Ems et al. 2011). Although the role of MyD88 in IL-10 production was not determined within our studies, there is still the possibility of either an unidentified surface TLR or that cooperation of multiple surface TLRs is responsible of Bb-elicited IL-10 production. Besides TLRs, other surface receptors, such
as the scavenger receptor CD36, are putative candidate receptors for recognizing Bb and elicitation of IL-10. CD36 was noted for its ability to recognize lipoproteins and to function synergistically with TLR2 (Hoebe, Georgel et al. 2005; Akashi-Takamura and Miyake 2008). It was also known to induce IL-10 production through p38 MAP kinase activation (Chung, Liu et al. 2007).

While the levels of IL-10 produced by macrophages in response to intact Bb remained the same with or without phagocytosis, the production of IL-10 in response to Bb-lipoproteins was significantly upregulated when phagocytosis was inhibited. One possible explanation is that extracellular TLR2 recognizes free Bb lipoproteins at greater affinity than internalized TLR2s. During phagocytosis, macrophages may internalize its surface receptors along with the microbial pathogen (Ozinsky, Underhill et al. 2000). A recent study demonstrated by confocal microscopy that TLR2s can be internalized and recruited to phagosomes upon interactions with Bb (Cervantes, Dunham-Ems et al. 2011). These findings indicate that there could be a loss of TLR2 or its ligands from phagocytes as they interact with Bb, resulting in a gradually reduced TLR2 signaling in response to Bb. Since TLR2 is expressed ubiquitiously on mammalian cells, it is likely that TLR2-expressing non-phagocytes as well as phagocytes can also produce IL-10 in response to Bb. Based on our finding that Bb-elicited IL-10 production is phagocytosis-independent, we speculate that TLR2-expressing non-phagocytic cells contribute to the source of the total host IL-10 produced as those cells capable of phagocytosis (i.e. APCs). Keratinocytes, which are known to potently produce IL-10, are also known to express high levels of TLR2 on their surface and are highly reactive against certain TLR2 agonists (Koller, Muller-Wiefel et al. 2011) (Ollert, Weissenbacher et al. 2005). It is also
possible that during the course of Bb-infection, pieces of Bb outer membrane can be shed off through a process known as membrane blebbing, resulting in increased concentration of free Bb lipoproteins within the host, which are capable of enhancing the elicitation of host IL-10. Certain spirochetes, such as members of the *Treponema* spps, have been shown to exhibit membrane blebbing (Cunningham, Walker et al. 1988; Baehni, Song et al. 1992). Therefore, whether Bb also blebs its outer membrane is in need of investigation.

Consistent with other studies, most Bb-elicited pro-inflammatory mediators, such as TNFα, require TLR2 signaling. However, since the production of pro-inflammatory mediators in response to Bb are phagocytosis-dependent, it is likely that the signals important for these responses originate from internalized TLR2 and may require the breakdown of Bb into individual TLR2 agonists. One interesting exception is IL-12 in that its production by macrophages in response to Bb was either unaffected or enhanced in the absence of TLR2 signaling. In addition, recombinant Bb lipoproteins induce very little to no IL-12 from macrophages. These findings further indicate receptors other than TLR2 participate in the immune sensing of Bb. Studies performed with Gram-positive bacteria *Lactobacteria* spps also indicated that similar to Bb, these bacteria induce IL-10 production by macrophages through TLR2. Similarly to Bb, the induction of IL-12 by *Lactobacteria* spps is also TLR2-independent and has been shown to be elicited by a distinct bacterial component from the ones responsible for induction of IL-10 (Kaji, Kiyoshima-Shibata et al.)(2010). This implies that Bb may also contain distinct structures that are responsible for eliciting different sets of immune responses from the host. It is interesting that Bb lacks most known characteristic components of a Gram-
positive bacteria (e.g. lipoteichoic acid), yet it displays similar patterns of host IL-10 and IL-12 elicitation. Taken together, there is still much to learn regards to how various Bb components interact with the immune system.

One conclusion from our current studies is that events associated with phagocytosis can significantly skew the type of immune response (pro- or anti-inflammatory) produced by macrophages in response to Bb. It is reasonable to believe that during the early stages of Bb infection, the bacteria invest most of its energy to avoid being phagocytosed by host immune cells. Intravital imaging studies within Bb-infected skins have revealed that during the initial 24 hours of Bb infection, the velocity of Bb was substantially greater than those of innate immune cells (Wooten unpublished). At this rate of Bb movement, it is likely that most of the Bb will avoid capture by phagocytic cells and remain in the extracellular milieau. Therefore, host immune response against Bb would be skewed toward one that is high in IL-10 production, but low in pro-inflammatory cytokines. This would also somewhat be reflected by the early symptoms of LD, where inflammatory symptoms exist but quickly abate, while the bacteria persist within the host. The phagocytosis-dependent elicitation of Bb-mediated pro-inflammatory can also be used to explain the high levels of tissue-specific inflammation associated with LD. Previous studies have indicated that an increase in IL-12 levels, and the subsequent upregulation of gamma-interferon present in serum and Bb-infected tissues, was directly proportional to LD disease severity (Anguita, Barthold et al. 2002; Hedrick, Olson et al. 2006). In addition, elevated levels of pro-inflammatory cytokines and chemokines were found in joint tissues of Bb-infected animals, as well as human LD patients (Shin, Glickstein et al. 2007; Wang, Ma et al. 2008; Shin, Strle et al. 2010).
From these thesis studies, we have shown that the elicitation of most pro-inflammatory responses against Bb by macrophages is phagocytosis-dependent. Therefore, it is likely that resident APCs are much more actively engaged in Bb-phagocytosis when Bb enters secondary tissues (e.g. knee joints) versus at the initial infection site. There are many possible explanations for this difference in Bb-elicited immune responses between the early and later stages of infection. For example, there could be a substantial decrease in Bb velocity when the bacteria reached denser secondary tissues versus while they were in the skin, thus allowing the resident APCs a better chance of capturing them. To assess this possibility in more detail, comparative studies involving quantifying the velocity of Bb within the secondary tissues versus the velocity of Bb while in skin are needed.

Better understanding the role Bb phagocytosis plays in generation of pro-inflammatory responses during the onset of LD can be beneficial for the development of therapies at different stages of the disease.

**The production of Bb-elicited IL-10 by macrophages utilizes PI3 kinase and MAP kinase pathways:** One goal for these thesis studies is to determine the intracellular signaling cascades involved in Bb-elicited IL-10 production and whether or not the roles of these pathways were distinct from the elicitation of pro-inflammatory responses. Our findings indicate that Bb and its lipoproteins strongly activate both PI3 kinase and MAP kinase pathways, including p38 and ERK1/2. *In vitro* analyses using specific inhibitors against these pathways demonstrated that Bb-elicited IL-10 production by macrophages is PI3 kinase, p38 MAP kinase, and ERK1/2-dependent. The activation of these pathways by macrophages in response to Bb were all found to be TLR2-
dependent, while largely independent of Bb-phagocytosis. In vivo studies also demonstrated that administration of a p38 inhibitor sub-cutaneously can suppress Bb-elicited IL-10 from the skin, leading to a reduction of Bb levels within the skin. These findings indicate that PI3 kinase and MAP kinases are important downstream pathways for the TLR2-mediated production of Bb-elicited IL-10 by macrophages.

Activation of PI3-kinase and AKT pathways have been known to down regulate excessive pro-inflammatory responses by immune cells (Schabbauer, Tencati et al. 2004). One such example involves the Fc receptor (FcR)-mediated signal transduction in macrophages. Studies indicated that prolonged stimulation through FcR results in enhancement of IL-10 production as well as suppression of IL-12 (Gerber and Mosser 2001). Subsequently, studies have verified that FcR-mediated IL-10 production by macrophages is dependent on PI3-kinase/AKT pathways (Polumuri, Toshchakov et al. 2007). Besides signaling through FcR, prolonged ligation of TLR agonists with its receptors can also induce IL-10 production through the activation of PI3-kinase/AKT pathways (Martin, Schifferle et al. 2003; Luyendyk, Schabbauer et al. 2008). Since Bb-specific antibodies were not used in the studies in Chapter 4 and that the Bb-lipoprotein OspA activated the PI3-kinase signaling pathway similar to that of intact Bb, it implies that it is TLR2, and not FcR, that initiate the PI3-kinase/AKT pathway-dependent production of IL-10 in response to Bb.

Since the PI3-kinase/AKT pathway plays a variety of roles in cell proliferation and thus regulates a variety of different cell signaling pathways (Cantley 2002), it is important to understand the holistic role of PI3-kinase/AKT signaling as it relates to the production of IL-10 by immune cells such as macrophages. In Chapter 4 of this thesis
study, the role of mammalian target of rapamycin (mTOR), a downstream target of the PI3-kinase/AKT pathways that has been shown to be involved in IL-10 production by macrophages (Weichhart, Costantino et al. 2008; Weichhart and Saemann 2008), was also examined in Bb-elicited IL-10 production. Our findings showed that inhibition of mTOR only partially suppressed Bb-elicited IL-10 production by macrophages, while no changes were observed in IL-12 levels. This indicates that mTOR is only one of many downstream mediators involved in the production of PI3-kinase/AKT-mediated cytokine production by macrophages in response to Bb. Recently, there have been studies indicating that macrophages deficient in PTEN, a phosphatase that opposes the activity of PI3-kinase, produces less IL-10 in response to Gram-negative bacteria compared to WT mice (Gunzl, Bauer et al. 2010). The same group also found that pathogen-induced PI3-kinase signaling results in the activation of a class of phosphatases known as DUSP1, which was described to possess various immune-suppressive activities within macrophages. Therefore, more studies regarding the role of PI3-kinase related molecule on Bb-elicited IL-10 are needed.

The MAP kinase pathway, known to be important for stress-related cellular responses, has also been implicated to be important for modulating innate immunity within macrophages (Huang, Shi et al. 2009). Likewise with the PI3 kinase/AKT pathway, MAP kinases such as ERK and p38 were also shown to be involved in FcR- or TLR-mediated IL-10 production by macrophages in response to pathogens or its components. (Kaji, Kiyoshima-Shibata et al.; Yang, Mosser et al. 2007). In addition, non-pathogen mediated IL-10 production, such as when macrophages respond to apoptotic cells, were also mediated through MAP kinase p38 (Chung, Liu et al. 2007). As for Bb,
there have been several reports indicating the activation of MAP kinase family members is important for the elicitation of host cytokines and matrix metalloprotease (Behera, Thorpe et al. 2004; Sahay, Patsey et al. 2009). Interestingly, the activation of MAP kinases was also proportional to the development of inflammation seen during Lyme arthritis, and that the source of the MAP kinase activation is from innate immune leukocytes (Anguita, Barthold et al. 2002). This may seem to be counter-intuitive to our findings that MAP kinases were also involved in the production of Bb-elicited IL-10. However, as aforementioned, the rate of Bb-phagocytosis may increase during latent stages of Bb-infection, which can shift immune response toward one that is high in production of certain pro-inflammatory cytokines (e.g. TNFα), which has also been shown to be MAP kinase-dependent. Additional studies are needed to further delineate the role of MAP kinase signaling cascade in the development of LD, as with PI3-kinase/Akt pathways.

While IL-10 production was dependent on PI3-kinase and MAP kinase pathways, the production of pro-inflammatory cytokines once again exhibited different trends. Bb-elicited IL-12 displayed the biggest disparity from IL-10, such that its levels were significantly enhanced in the absence of either PI3 kinase or MAP kinase signaling. This trend, along with our finding that IL-12 elicitation was phagocytosis-dependent yet TLR2 independent, demonstrated that pathways involved in the production of IL-12 are most different from IL-10 than those of other cytokines. This is expected, since IL-10 and IL-12 exerts opposing functions within the host. Others have indicated that inhibition PI3 kinase or MAP kinase activity enhanced IL-12 production in response to various macrophage agonists, while IL-10 production decreased (Chelvarajan, Popa et al. 2007;
Polumuri, Toshchakov et al. 2007; Yang, Zhang et al. 2010). Bb-elicited IL-6 production by macrophages, which was demonstrated in Chapter 3 to follow the same surface signaling as Bb-elicited IL-10, was shown in Chapter 4 to be independent of either PI3 kinase or MAP kinase. Therefore, it is possible that upon the sensing of Bb on the surface of macrophages, the signaling cascade is split two ways, one for IL-10 and another for IL-6. For all the pro-inflammatory cytokines assessed, TNFα production in response to Bb is most similar to IL-10 in terms that it is PI3 kinase- and MAP kinase-dependent. It has been observed that *Leishmania*-elicited TNFα production by macrophages were also dependent on p38 MAP kinase (Yang, Zhang et al. 2010). As for Bb, others have also indicated that TNFα production in response to Bb from CD14−/− macrophages is PI3 kinase dependent, yet independent of p38 (Sahay, Patsey et al. 2009). Therefore, it is likely that the production of Bb elicited IL-10 and TNF shares the same intracellular signaling cascade, although they differ at the level of cell surface recognition such that IL-10 production is phagocytosis-independent while TNFα is not. Since TNFα is usually regarded as the first cytokine to be upregulated by activated macrophages in response to microbial agonists, the fact that Bb-elicited IL-10 production shares some common signaling pathway as TNFα could explain why Bb is able to upregulate host IL-10 at such a rapid rate.

One conclusion from this thesis work is that Bb elicits both PI3-kinase and MAP kinase signaling cascades from host cells (e.g. macrophages) upon infection, and this elicitation results in upregulation of IL-10 production while down-regulating IL-12 production by APCs such as macrophages. Previous findings have indicated that Bb-mediated signaling through TLR2 can lead to the desensitization of macrophages through
an IL-10 dependent manner (Diterich, Rauter et al. 2003). Since PI3-kinase and MAP kinases were downstream mediators of Bb-induced TLR2 activation and that they play a role in IL-10 production, these pathways may participate in the induction of Bb-induced macrophage tolerance. Studies were performed to test whether administration of inhibitors against one of these pathways (i.e. MAP kinase p38) \textit{in-vivo} will result in a shift toward greater pro-inflammatory responses against Bb. Findings from Chapter 4 indicated that mice injected with specific p38 MAP kinase inhibitor (i.e. SB203580) prior to Bb infection yielded significant reduction of IL-10 levels in skin tissues 30 hours post-Bb infection. However, the levels of pro-inflammatory cytokines assessed (e.g. TNF\(\alpha\)) remained unchanged. One limitation with the study was that although other cytokines such as IL-12 and IL-6 were assessed, their levels were below the detectible limits, suggesting that they were either not produced this early during skin infection or a more sensitive method of detecting pro-inflammatory cytokines \textit{in-vivo} is needed. The pleitropic effects of the PI3 and MAP kinase pathways may indicate that other cellular activities besides cytokine production may be regulated within the host in response to Bb-infection. Therefore, studies such as a microarray analysis of potential downstream effectors of the two pathways within Bb-infected skins need to be performed.

\textbf{Integrated themes from thesis work:} The overall findings from the three chapters of this thesis work, along with previous studies, allow us to better model the role of IL-10 during early stages of Bb-infection. Upon the immediate entry into the host skin, Bb is recognized by resident APCs, such as macrophages and DCs, through the sensing Bb lipoproteins by TLR2 expressed on the surface of APCs. This surface interaction
between Bb and host cells subsequently activates downstream signaling cascades, such as PI3-kinase/Akt, MAP kinases p38, and ERK1/2 MAP kinase pathways, from APCs. The activation of these signaling cascades can modulate APCs response against Bb, including dysregulating the production of host IL-10. As a result of this aberrant wave of IL-10, various host innate immune activities, including chemokine production, respiratory burst, phagocytosis, and possibly the recruitment/activation of neutrophils at site of Bb infection are suppressed. In addition to dampening the innate immune responses against the bacteria, Bb-elicited IL-10 can possibly suppress the adaptive immune response through the suppression of IL-12 and MIP1α production, as well as down-regulation of surface co-stimulatory molecule such as CD86 by APCs. Ultimately, the sub-optimal immune responses elicited against Bb as a result of the dysregulated IL-10 levels allows the bacteria to better evade immune clearance and thus allowing the dissemination of Bb toward target tissues that are immune-privileged.

The knowledge obtained on how Bb manipulates host IL-10 to promote persistence can be applied toward designing more effective therapeutics against Lyme disease and Bb infection. Although antibiotics against Bb exist and are quite effective in eventually clearing Bb, there are still adverse affects associated with antibiotics. For example, it is possible for Bb to develop antibiotic resistance. In fact, studies have revealed that Bb expresses drug efflux pumps on its outer membrane (Bunikis, Denker et al. 2008). Since most of these antibiotics are not Bb-specific, prolonged administration of these agents may induce detrimental side-effects (e.g. induction of antibiotic resistance from other bacteria). An alternative form of therapy against Bb infection is to bolster the host immune system toward better clearance the pathogen. The advantages of developing
immune-enhancing therapies is that 1) it does not place selective pressure directly on the pathogen so drug resistance is less likely to occur, and 2) multiple pathogens that share a common method of immune-evasion can be targeted using the same treatment. As far as Bb is concerned, therapeutics targeting the production or the effects of dysregulated IL-10 levels should enhance the immune clearance of the bacteria. A potential method is to administer blocking antibodies against either IL-10 or its receptors systemically. Studies have shown that the administration of blocking antibodies against IL-10 receptor can reduce the persistence of certain retroviruses, such as lymphocytic choriomeningitis virus (LCMV), within infected animals (Brooks, McGavern et al. 2006). However, the systemic depletion of host IL-10 activity can also lead to an exacerbated level of pathogen-induced inflammation within the host (Sewnath, Olszyna et al. 2001). Therefore, the administration of neutralizing antibodies against either IL-10 or IL-10 receptor to Bb-infected patients is often risky since it may increase their susceptibility to developing inflammatory diseases (e.g. sepsis) when exposed to a second infectious agent during the course of the treatment.

An alternative immune therapeutic strategy against Bb is to selectively target the host cells and the intracellular signaling pathways specific for production of Bb-elicited IL-10, while leaving other IL-10 producing cells/mechanisms unaffected. In this thesis work, we have identified that p38 MAP kinase is one of the pathways involved in Bb-elicited IL-10 production by macrophages. Therefore, targeting p38 MAP kinase activation within infected host may lead to improved Bb clearance. In Chapter 4, we demonstrated that administration of the p38 MAP kinase inhibitor SB203580 into Bb-infected wt mice over a three-week interval results in significantly lower Bb loads within
the skin compared to WT mice administered with PBS (controls), corresponding to Bb levels similar to those that persisted within the IL-10⁻/⁻ mice. However, the reduced levels did not achieve statistical significance in other Bb target tissues assessed (e.g. heart, ankle, and ear), although the levels were similar to those observed in IL-10⁻/⁻ mice. This finding indicates many possibilities regarding the role of administering p38 MAP kinase inhibitors as potential therapies to promote Bb clearance. First, p38 MAP kinase may only affect Bb-elicited IL-10 production by cells within the skin, whereas cells outside of skin utilize other mechanisms for IL-10 production. Second, the inhibition of p38 MAP kinase may inadvertently inactivate certain immune responses that are actually important for Bb clearance, resulting in cancelling out the effect of Bb clearance by suppressing IL-10 elicitation alone, which would explain why in certain tissues, the level of Bb load in IL-10⁻/⁻ mice administered with SB is actually greater that those of IL-10⁻/⁻ controls. For example, a study demonstrated that the administration of p38 inhibitor to Bb-infected mice over a period of two weeks have resulted in suppressed T cell activities against Bb (Hedrick, Olson et al. 2006). Although administration p38 MAP kinase inhibitor showed some promises as ways to boost immune clearance against Bb mainly during the early stages of infection, much work are still needed to understand the relevance of p38 MAP kinase in the immune responses against Bb. Since both PI3-kinase and p38 MAP kinase are key signaling pathways for many pathogen-elicited IL-10 production, developing therapeutics that regulates their activities for host IL-10 production may be beneficial to control multiple infectious agents simultaneously. In fact, it has been shown in Leishmania infections that administering ERK inhibitors in-vivo to suppress pathogen-induced IL-10 yielded significant improvement in the
clearance of the parasite (Yang, Mosser et al. 2007). However, both PI3-kinase and MAP kinase signaling are also involved in various cellular activities besides IL-10 production, which implies that manipulation of their functions may lead to adverse side-effects. Therefore, further studies examining the downstream effectors from these pathways that are more specific toward IL-10 production in response to Bb is needed.

Besides developing therapeutics that interferes with the cellular pathways of IL-10 production, an alternative approach would be to reverse the suppressed immune responses mediated by Bb-elicited IL-10. For example, developing agents that will boost neutrophil recruitment, increase ROS generation, and enhance CD86 surface expression could all promote better Bb clearance. Since cytokines such as IL-12 and gamma interferon are known to exert opposing effects to IL-10, finding ways to elicit these cytokines within the host can boost resistance to Bb infection (i.e. as prophylaxis). Agents that will enhance the phagocytosis of Bb by immune cells can be beneficial for Bb clearance since one, it can increase direct killing and two, it can lead to upregulation of pro-inflammatory cytokine production by the infected host to counter-balance the level of Bb-elicited IL-10. Finally, identifying the candidate Bb components most responsible for IL-10 elicitation (i.e Bb lipoproteins) can also be beneficial to enhancing immune clearance against Bb such that neutralizing agents against these components can be administered to infected hosts.

I believe the findings from this thesis work have advanced our knowledge of host immune responses against Bb in the context of IL-10. However, there are still studies that need to be performed to further the field, some of which has been mentioned throughout this chapter. There are still many potentially important innate immune
effectors against Bb that needs to be investigated. Recently, the enzyme 5-lipoxygenase, the principal enzyme required for the generation of leukotrienes, has been implicated in Lyme arthritis development (Blaho, Zhang et al. 2011). Although macrophages are known to generate many of these inflammatory lipids, whether these lipids are generated in response to Bb and whether IL-10 plays a role in their generation needs to be investigated. There is also a need to determine the host cell types that are the principle producers of IL-10 in response to Bb infection. As aforementioned, other cell types that normally reside within the skin are the most plausible candidate, since this is where Bb usually enters the host. However, IL-10 producing cells that reside outside of skin are also important. One such cell type are marginal-zone B cells, a B cell subset usually found within the marginal zone of the spleen, which are known for potent IL-10 production (Lenert, Goeken et al. 2003) and have been indicated to be important in Bb immunity (Belperron, Dailey et al. 2007). The findings from Chapter 4 that p38 MAP kinase inhibitor only enhanced Bb clearance within the skin while IL-10−/− mice showed enhanced Bb clearance in all tissues further emphasizes a need to delineate IL-10 elicitation patterns as Bb disseminates to their target tissues. Transgenic mice that express GFP-IL-10, such as the TIGER mouse (Kamanaka, Kim et al. 2006), may be beneficial for tracking the concentration gradient of IL-10 produced within a Bb-infection host. Together with our current findings, these perspective experiments should continue to expand our understanding on how Bb utilizes host IL-10 to achieve persistence in immunocompetent hosts.

Identifying how host IL-10 plays a significant role in Bb clearance is a major step towards deciphering the mechanisms of how Bb achieves persistence. This
understanding also opens new doors for studies regarding host responses against Bb. Characterizing the suppressive functions of Bb-elicited IL-10 on the cellular level will allow determination of which immune effectors are most important for Bb clearance within immune-competent hosts. Better understanding of critical pathways involved in the production of IL-10 by host cells in response to Bb will also be beneficial in the designing of better therapeutics to treat LD and limit Bb persistence. Finally, the knowledge gained on how host IL-10 is elicited by Bb may serve as a model system for understanding the role host IL-10 played in host-pathogen interaction in other infectious diseases.
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